# DEVELOPMENTS IN THE MUTANT PREVENTION CONCENTRATION:

## A novel approach to antimicrobial susceptibility/resistance issues

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By

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## ABSTRACT

The mutant prevention concentration (MPC) is defined as the lowest antimicrobial concentration required to inhibit the growth of the least susceptible bacterial cell based on an inoculum of $\geq 10^9$ colony forming units (CFUs). The current protocol for MPC testing is technically demanding and time-consuming which limits its implementation into clinical microbiology laboratories. In an attempt to simplify the current MPC protocol we developed a modified MPC method, the microbroth dilution method, which requires two fewer days to complete than the current or traditional method. MPC values were consistent for all organisms and strains tested using both the traditional MPC method and the modified microbroth dilution MPC method.

Tigecycline is the first of a new class of compound – glycylcyclines- with potent *in vitro* activity against Gram-positive organisms including penicillin-resistant and multi-drug resistant *Streptococcus pneumoniae* (SP) and methicillin-resistant *Staphylococcus aureus* (MRSA). We measured minimum inhibitory concentration (MIC) and MPC values for tigecycline against 47 clinical isolates of SP and found that the $MPC_{90}$ values were >500 fold higher than the $MIC_{90}$ values. To determine if MPC testing of tigecycline against SP is impacted by blood in the medium, we developed a new medium able to sustain the growth of SP without the need for blood; solidified Todd-Hewitt broth (sTHB). The $MPC_{90}$ values of tigecycline against SP on sTHB were only 2 fold higher than the $MIC_{90}$ values. When blood was added to the sTHB, the $MPC_{90}$ values again became much greater than the $MIC_{90}$ values (> 256 fold higher). MPC results for *Staphylococcus* spp. against tigecycline were not impacted by blood in the medium.

Benzalkonium chloride (BAK) is a cationic surface-acting agent that acts on bacterial cells by disrupting the intermolecular interaction of the lipid bilayer. To determine if the fluoroquinolones gatifloxacin (Gfx) and moxifloxacin (Mfx) are more active (lower MIC values) in the presence of BAK, we conducted MIC, MPC, and time-kill assays. MIC testing showed that in the presence of 3.125 to 50 μg/ml of BAK, the MIC of Gfx and Mfx decreased by 8- to 5000-fold against clinical isolates of methicillin-susceptible *Staphylococcus aureus* (MSSA), MRSA, Coagulase-negative Staphylococci (CNS), SP, *Escherichia coli* (EC), and *Pseudomonas aeruginosa* (PA). MPC testing showed that the presence of 7 to 10 μg/ml of BAK, the MPC of Gfx and Mfx decreased by 32- to 1000-fold against clinical isolates of MRSA. Conventional time-kill studies (using a bacterial load of $10^5$ CFUs) showed that the killing activity of Gfx against clinical MRSA isolates was enhanced in the presence of BAK with a $\log_{10}$-reduction (percent kill) of 1.6 (76.08%) for Gfx alone at 180 minutes compared to a $\log_{10}$-redecution (percent kill) of 5.4 (100%) for Gfx plus BAK at 180 minutes.

Alexidine (Alx) is a bisbiguanide that has been used as an effective disinfectant in the dental industry and is potentially being developed for use as an antimicrobial agent for ocular infections. We conducted susceptibility testing of Alx using MIC testing, MPC testing, and time-kill assays against Gram-positive and Gram-negative pathogens. MIC testing showed that Alx is more active against Gram-positive pathogens than Gram-negative pathogens and showed better activity than the fluoroquinolones Gfx, Mfx, and levofloxacin (Lfx) against MRSA. The MPC values measured for MRSA and MSSA against Alx were non-reproducible using the traditional MPC method. Using the microbroth dilution MPC method, $MPC_{90}$ values were found to be 32 fold higher than the

$MIC_{90}$ values. If the experimentally determined MPC values are "true" MPC values, initial MPC testing indicates that Alx may have a high likelihood for selecting for resistance, however, if the MPC values are not accurate it may be necessary to modify the MPC protocol in order to complete MPC testing of Alx against MRSA and MSSA. Conventional time-kill studies (using a bacterial load of $10^5$ CFUs) measured bactericidal activity (> 3 $\log_{10}$-reduction) against MRSA, MSSA, SP, and PA.

## ACKNOWLEDGEMENTS

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| | |
|---|---|
| Alx | Alexidine |
| ATCC | American Type Culture Collection |
| BAK | Benzalkonium Chloride |
| CBSN | Canadian Bacterial Surveillance Network |
| CLSI | Clinical and Laboratory Standards Institute |
| CNS | Coagulase-negative staphylococci |
| CFU | Colony Forming Units |
| DNA | Deoxyribonucleic acid |
| EC | *Escherichia coli* |
| Gfx | Gatifloxacin |
| HI | *Haemophilus influenzae* |
| Lfx | Levofloxacin |
| MSSA | Methicillin-susceptible *Staphylococcus aureus* |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| µg | Microgram |
| µl | Microlitre |
| ml | Mililitre |
| MIC | Minimum Inhibitory Concentration |
| Mfx | Moxifloxacin |
| MH | Mueller Hinton |
| MHB | Mueller Hinton Broth |
| MPC | Mutant Prevention Concentration |
| MSW | Mutant Selection Window |
| O.D. | Optical Density |
| PA | *Pseudomonas aeruginosa* |
| QAC | Quaternary Ammonium Compound |
| QRDR | Quinolone Resistance Determining Region |
| RNA | Ribonucleic acid |
| rpm | Rotations Per Minute |
| SRBC | Sheep Red Blood Cells |
| sTHB | Solidified Todd Hewitt Broth |
| SP | *Streptococcus pneumoniae* |
| THB | Todd Hewitt Broth |
| TSA | Tryptic Soy Agar |

## 1.0 INTRODUCTION

### 1.1 Antimicrobial Resistance

With the initial discovery and introduction of Penicillin G in the early 1940s the pre-antibiotic age ended; an age when simple bacterial infections often meant life-threatening disease and death [1]. Unfortunately, by 1944 antimicrobial resistance had already appeared with strains of penicillin-resistant *Staphylococcus aureus* being reported [2]. With great foresight, Alexander Fleming, who won the Nobel Prize in 1945 for the discovery of penicillin, warned in 1946, "…the greatest possibility of evil in medication is the use of too small doses so that instead of clearing up infection the microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out, which can be passed to other individuals and from them to others, until they reach someone who gets septicemia or pneumonia which penicillin cannot save" [3]. Unfortunately, we did not heed Fleming's warning and nearly sixty-five years later, virtually all strains of *Staphylococcus aureus* are resistant to natural penicillins [2]. According to the National Nosocomial Infections Surveillance (NNIS) system report for 2004, 59.5% of *Staphylococcus aureus* strains in intensive care unit (ICU) patients were methicillin-resistant [2, 4]. According to the Canadian bacterial surveillance network (CBSN) the prevalence of penicillin-resistant *Streptococcus pneumoniae* was virtually non-existent in the early 1980s, but by 2005, 15% of *Streptococcus pneumoniae* isolates were penicillin-resistant [5]. Unfortunately the development of antimicrobial resistance is not limited to penicillins; in fact, bacterial evolution has culminated in the emergence of resistance to every approved antibiotic [1, 2, 6, 7]. Antimicrobial resistance was recently described by Blondeau *et al* as a global pandemic [8-10]. This definition seems fitting as antimicrobial

resistance is a growing concern worldwide with more and more bacterial organisms developing resistance to an ever-increasing number of antimicrobial agents [11, 12]. Resistance rates are increasing among Gram-positive and Gram-negative organisms and the increase in multi-drug resistant (MDR) organisms is very troubling as they are increasingly difficult to treat [1, 2, 6, 7, 11, 13]. Reported rates of MDR isolates from intensive care units in the U.S. increased from 4% in 1993 to 14% in 2002 and rates of MDR *Pseudomonas aeruginosa* increased from 12.8% in 1997 to 20.8% in 2000 [11].

**1.2 Factors Leading to Antimicrobial Resistance**

Virtually all genera and species of pathogenic bacteria are either innately resistant or acquire resistance to antimicrobial agents [9]. For organisms to be innately resistant to a particular antimicrobial agent (or class of antimicrobial agents) they must possess an inherited trait that allows them to resist the killing or inhibitory effect of the antimicrobial agent(s). Of greater concern are organisms which have acquired resistance; where a bacterial population initially susceptible to an antimicrobial agent or class has become resistant to that agent allowing the population to proliferate and spread under the selective pressure of that agent [9, 14]. One of many ways bacteria acquire resistance is through the acquisition of genes encoding enzymes that destroy the antimicrobial agent before it can have an effect. These genes can be acquired by any one of several genetic mechanisms, including transduction, transformation, and conjugation and because of these genetic mechanisms it is not uncommon for some organisms to have several resistance mechanisms, potentially conferring on them resistance to multiple classes of antimicrobial agents [9, 14]. Bacteria that are resistant to three or more antimicrobial

classes are defined as multi-drug resistant (MDR) organisms and are of serious concern because of their potential difficulty to be treated [14].

Spontaneous mutation and the acquisition of genes encoding efflux pumps are two additional ways in which bacteria are able to resist the effects of antimicrobial agents. Efflux pumps function by expelling the antimicrobial agent from the bacterial cell before it can exert its biological effect while spontaneous mutations are able to confer resistance many different ways including, the alteration of target proteins to which antimicrobial agents bind [14]. While it is highly unlikely that a single genetic mutation would result in high-level resistance to an antimicrobial agent, it may allow the bacterial cell containing the mutation to survive long enough to acquire additional mutations or resistance mechanisms [15]. There are reports, although rare, of high-level antimicrobial resistance caused by a single mutation [14].

There are several factors that have been attributed to causing or have been associated with antimicrobial resistance. These include but are not limited to the following: over-prescription of antimicrobials, the use of inappropriate antimicrobials, incorrect dosage of antimicrobials, veterinary use of antimicrobials, clinical trials excluding patients with resistant pathogens, and clinical trials which focus solely on clinical outcomes [8]. While there is little doubt that all of these factors contribute to antimicrobial resistance, it is widely accepted that the main causes have been, and still are widespread inappropriate use and over-prescribing of antimicrobial agents in clinical practice [7, 16]. In a study conducted by Rybak and associates, indiscriminate antibiotic treatment was found by looking at 100 consecutive patients prescribed fluoroquinolones in the emergency departments of two institutions and subsequently discharged. After

reviewing the appropriateness of these prescriptions, Rybak and associates determined that 81 of the 100 patients received a fluoroquinolone inappropriately. Of the 19 patients treated appropriately with a fluoroquinolone (as determined by guidelines and cause of infection), only one was prescribed the correct dosage and treatment duration [17]. In fact, data in the US indicate that at least half of patients diagnosed with infections caused largely by respiratory viruses received antibiotic therapy [18].

**1.3 Ocular Infections**

Common ocular infections such as conjunctivitis, keratitis, and endophthalmitis are caused by a diverse group of bacterial, viral, and fungal pathogens and treatment therefore includes the use of antivirals, antifungals, and antibacterial agents [19]. Bacterial keratitis is the leading cause of blindness in the developing world while conjunctivitis is one of the leading causes of visits to the emergency room or doctor's office in the developed world [20-23]. As contact-lenses become more popular, the incidence of bacterial keratitis has significantly increased with current estimates reflecting 10-30 cases per 100,000 people using contact lenses annually [21]. Gram-positive cocci, especially *Staphylococcus* spp., are by far the most common cause of bacterial ocular infections [24, 25]. Other important bacterial organisms involved in ocular infections include *Streptococcus pneumoniae*, *Haemophilus* spp., *Moraxella* spp., *Corynebacterium diphtheriae*, and *Neisseria* spp. [21].

Three fluoroquinolones were introduced in the 1990s for topical use of common bacterial infections such as conjunctivitis and keratitis; these fluoroquinolones were ciprofloxacin 0.3%, ofloxacin 0.3%, and norfloxacin 0.3% [26]. Fluoroquinolones were welcomed in the field of ophthalmology because of their good penetration and relatively

broad-spectrum activity; they were also shown to be equivalent to combination therapy in the treatment of many ocular infections [19, 27, 28]. Unfortunately, since their introduction, *in vitro* resistance to these three fluoroquinolones has been steadily rising among both Gram-positive and Gram-negative organisms [22, 26, 29-31]. In a 16 year study conducted by Davis *et al* and later summarized by Blondeau, significant resistance was found to ciprofloxacin and ofloxacin; 606 bacterial endophthalmitis isolates were analyzed and in 1994, 1997, and 2000 the percentage of *Staphylococcus aureus* isolates resistant to ciprofloxacin was 32%, 42%, and 67% respectively [24, 31]. Similar trends were seen in resistance to ofloxacin [24, 31].

Three new fluoroquinolones were recently introduced and approved for topical for ocular indications; levofloxacin 0.5% in August 2000, gatifloxacin 0.3% in March 2003, and moxifloxacin 0.3% in April 2003. These new fluoroquinolones have enhanced Gram-positive activity (lower MIC values) when compared to the older fluoroquinolones ciprofloxacin and ofloxacin [26, 32]. Gatifloxacin and moxifloxacin use a unique dual-binding mechanism of action in Gram-positive organisms and, therefore, represent the most advanced group of compounds within the fluoroquinolone class [19]. Not only are these two antimicrobial agents more active against Gram-positive organisms than previous agents, but they also provide less opportunity for the emergence of fluoroquinolone-resistant populations [28]. The addition of a unique bicyclic side chain at C-7 on the molecular structure decreases the risk for resistance by inhibiting the bacterial efflux pump mechanism [19, 26, 33]. Unfortunately, despite their advanced structure and better spectrum of activity, there have been recent reports of emerging resistance against gatifloxacin and moxifloxacin [28, 34].

## 1.4 Fluoroquinolones

The first quinolone antibiotic, nalidixic acid, was introduced in 1962 and was an antibiotic by-product in the synthesis of chloroquine [35]. Nalidixic acid had good *in vitro* antibacterial activity against Gram-negative cocci and therefore was of clinical use in uncomplicated urinary tract infections [36]. In an effort to improve the spectrum of activity of nalidixic acid, several structural modifications were made which ultimately resulted in four generations of quinolones, each with a broader spectrum of *in vitro* antibacterial activity (Figure 1.1) [28, 35]. The second generation quinolones were introduced in the 1980s, and included ciprofloxacin, norfloxacin, and ofloxacin. The second generation fluoroquinolones were developed by adding a fluoro-group at position 6 resulting in limited antimicrobial activity against Gram-positive organisms while retaining good antimicrobial activity against Gram-negative organisms [35]. These compounds are referred to as fluoroquinolones.

The classification of third and fourth generation fluoroquinolones is somewhat unclear. From a systemic treatment perspective, antimicrobial agents such as levofloxacin, gatifloxacin, and moxifloxacin are third generation fluoroquinolones [37]. However, in the field of ophthalmology, levofloxacin is considered a third generation fluoroquinolone while gatifloxacin and moxifloxacin are both considered fourth generation fluoroquinolones [19, 28]. The newer fluoroquinolones contain modifications at the C-5, C-7, and C-8 positions which further enhance their Gram-positive activity. The introduction of the $NH_2$ group at C-5, the alkylation of the piperazine group at C-7, and the addition of a methoxy group at C-8 all resulted in an increase in anti-Gram-positive activity [35]. The newer fluoroquinolones therefore exhibit good *in vitro*

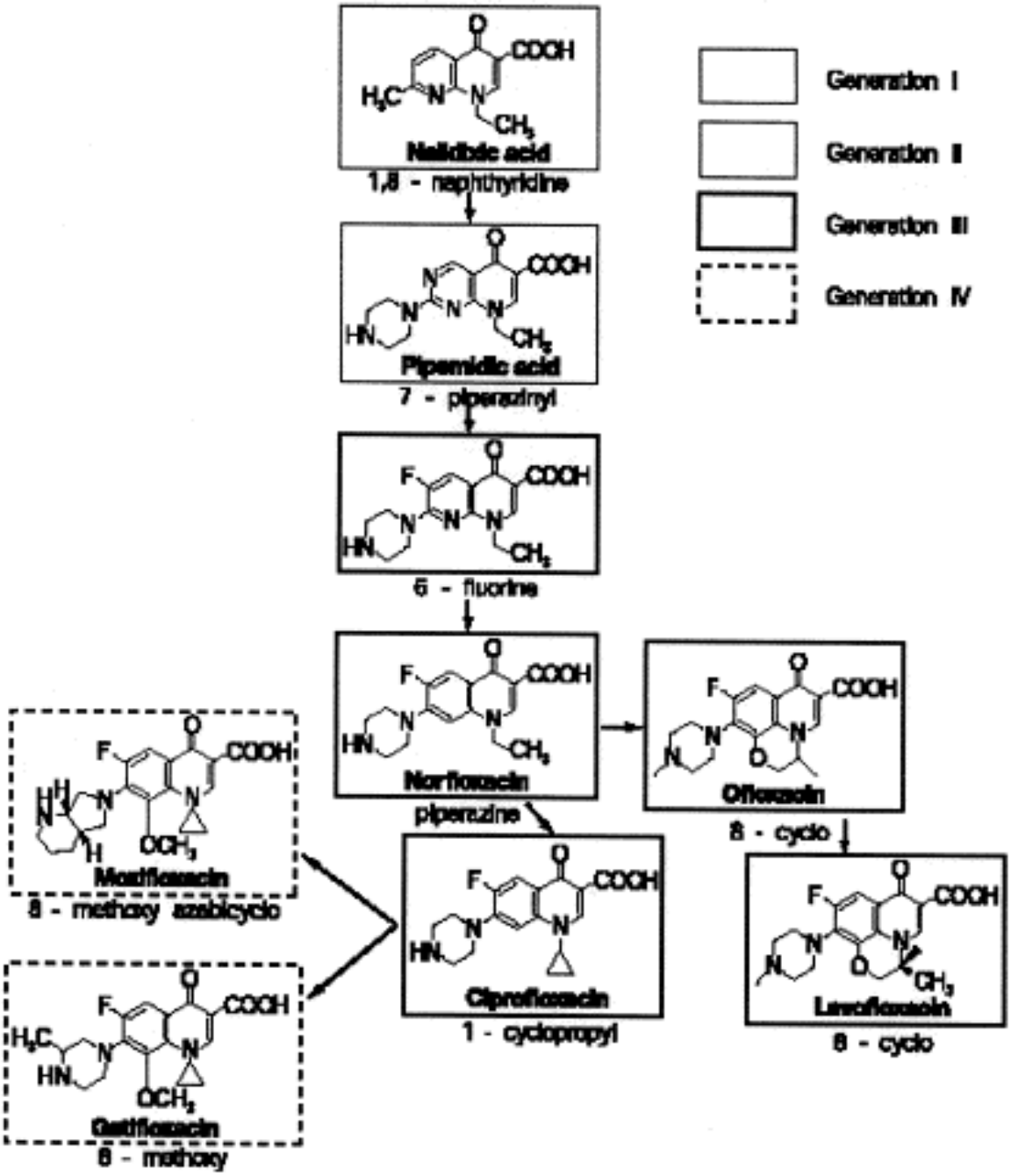


**Figure 1.1**: Structural Evolution of the Fluoroquinolones [19].

antimicrobial activity against Gram-positive and Gram-negative organisms [19, 27].

### 1.4.1 Mechanism of Action

The fluoroquinolones act on bacterial cells by inhibiting two enzymes involved in bacterial DNA synthesis; DNA gyrase and topoisomerase IV. Human cells lack both of these enzymes therefore enabling these agents to be specific [19, 27]. DNA gyrase is responsible for introducing negative supercoils into the DNA double helix ahead of the replication fork resulting in the separation of replicated daughter chromosomes [19, 23, 38, 39]. DNA gyrase is composed of two monomeric subunits, GyrA and GyrB, encoded by the *gyrA* and *gyrB* genes respectively. Topoisomerase IV segregates daughter chromosomes at the end of a round of replication in a process known at decatenation. Decatenation involves removing the interlinking of daughter chromosomes after replication; it also results in relaxation of the supercoiled DNA [19, 27]. Topoisomerase IV is composed of four homologous monomeric subunits, two ParC subunits and two ParE subunits, encoded by the *parC* and *parE* genes respectively. The structure and function of DNA gyrase and topoisomerase IV are detailed in Figure 1.2. When fluoroquinolones interact with the DNA-enzyme complex, they create a conformational change that results in the inhibition of normal enzyme activity. DNA synthesis is ultimately inhibited and the bacterial cell experiences rapid bacterial cell death [19].

For the older fluroquinolones, DNA gyrase is the target enzyme for most Gram-negative organisms while topoisomerase IV is the target enzyme for most Gram-positive organisms [27]. An important feature of the newer fluoroquinolones, such as gatifloxacin and moxifloxacin, is their dual activity (i.e. they are able to act on both DNA gyrase and topoisomerase IV) [41]. This is an important feature; dual activity fluoroquinolones

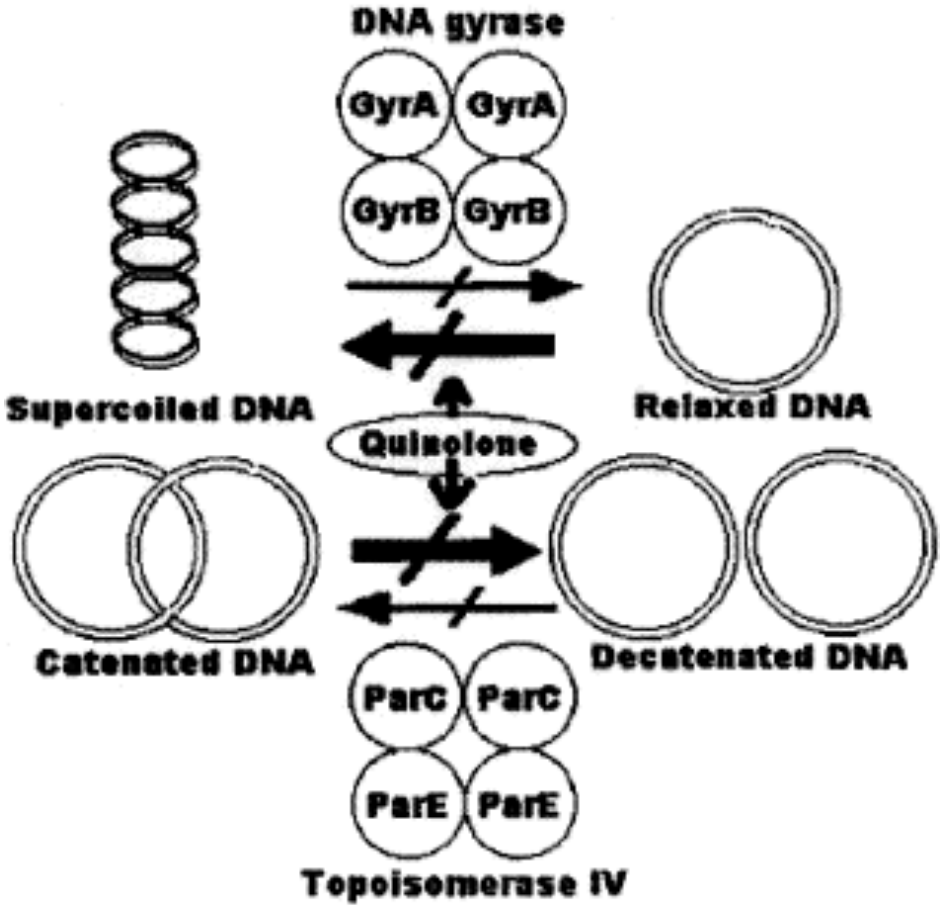


**Figure 1.2**: Structure and principal function of DNA gyrase and topisomerase IV [40].

require a bacterial isolate to contain a double mutation (mutations in each of the *parC* and *gyrA* genes) in order to resist their antimicrobial effect.

### 1.4.2 Mechanisms of Resistance

The fluoroquinolones are subject to various mechanisms of resistance, including, point mutations in *gyrA* or *gyrB*, point mutations in *parC* or *parE*, membrane-associated efflux proteins (*norA*), and a locus (*flqA*) which confers quinolone resistance in *Staphylococcus aureus* [27]. Point mutations in DNA gyrase are most commonly found in Gram-negative organisms, whereas point mutations in topoisomerase IV are most commonly found in Gram-positive organisms [40]. These point mutations are most commonly seen in areas of the bacterial genome known as quinolone resistance determining regions (QRDRs). The single target action of older fluoroquinolones makes them especially vulnerable to single mutations and as such single mutations are able to significantly affect the potency of these older drugs. Due to the dual activity of the newer fluoroquinolones, single mutations have little effect on the potency of these newer fluoroquinolones (i.e. gatifloxacin and moxifloxacin) [41].

Efflux-mediated resistance is a growing problem in the quest to develop potent antimicrobial agents and efflux mechanisms have been shown to make a particularly important contribution to fluoroquinolone resistance [27]. The main gene involved in bacterial-mediated efflux of fluoroquinolone antibiotics is the *norA* gene [27]. Dependent on the proton motive force, the *norA* gene mediates the efflux of drug from the bacterial cell, although its exact mechanism of action is unclear [42].

## 1.5 Novel Antimicrobial Agents

There have been only a few new antimicrobial agents approved for use in the last ten years and even more concerning is the fact that very few new antimicrobial agents are in the pipeline to be marketed in the future [11]. Since 1998 only ten new antibacterial agents have been approved by the U.S. Food and Drug Administration (FDA) and only three of these had novel mechanisms of action; linezolid, daptomycin, and tigecycline (approved in 2000, 2003, and 2005 respectively). Out of the three antibacterial agents with novel mechanisms of action only tigecycline has a broad spectrum of activity [43]. Shockingly, approval of new antibacterial agents by the FDA decreased by 56% from 1983-1987 to 1998-2002 [17]. We are in dire need for new antimicrobial agents, particularly with different mechanisms of action [14, 43].

### 1.5.1 Cationic Antimicrobial Agents

Cationic antimicrobial agents have been used for over a century in both infection control and within many consumer products including: pool/hot tub disinfection products, biocidal bandages, hair care products, baby diapers, inks, and mouthwash/dental preparations [44]. Two classes of cationic antimicrobial agents that have been in use for over 40 years include the quaternary ammonium compounds (QACs) and the bisbiguanides [16]. Bisbiguanides are well known anti-plaque agents and play a critical role in the reduction of supragingival plaque and treatment of gingivitis, while QACs are used extensively in the food processing industry to prevent the persistence of pathogens such as *Escherichia coli* and *Listeria monocytogenes* [16, 45-47]. Two antimicrobial agents discussed in the next section, benzalkonium chloride (BAK) and alexidine are QACs and bisbiguanides.

Cationic antimicrobial agents are bactericidal agents that exert their effect by binding to the bacterial cell surface and integrating themselves into the cytoplasmic membrane. This cellular disruption is generally sufficient to cause the membrane to lose fluidity and for the bacterial cell to die [16]. All that these cationic agents require to interact with a bacterial cell surface is a strong positive charge together with a hydrophobic region. The strong positively charged cationic molecules are able to bind to bacterial cells because of the opposite negative charge of bacterial cell walls. Bisbiguanides carry two cationic groups while QACs are generally monocationic [16].

#### 1.5.1.1 Benzalkonium chloride

Benzalkonium chloride (BAK) is a cationic antimicrobial agent belonging to the quaternary ammonium compound (QAC) family. It is currently being used as a preservative in the commercial preparation of Zymar® (0.3% gatifloxacin plus 0.005% BAK). Zymar®, a registered tradename of Allergan, Inc., is a topical ophthalmic solution indicated for treatment of bacterial conjunctivitis caused by susceptible strains of *Corynebacterium propinquum*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mitis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. Recent studies indicate that BAK used in commercial preparations, such as Zymar®, may not only act as a preservative, but may also contribute to a clinical effect [48]. Our lab has shown that the addition of BAK to gatifloxacin or moxifloxacin results in a reduction of the MICs provided by gatifloxacin or moxifloxacin without BAK [24, 49, 50].

In describing the action of QACs such as BAK, Salton and associates proposed the following sequence of events: (i) adsorption and penetration of the agent into the bacterial cell wall; (ii) reaction with the cytoplasmic membrane followed by membrane

disorganization; (iii) leakage of intracellular low-molecular weight material; (iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes [51]. A cartoon detailing the mechanism of action at the molecular level is shown in Figure 1.3. First, the positively charged quaternary nitrogen associates with the head groups of phospholipid bilayer within the membrane (Figure 1.3b). The hydrophobic tail then interdigitates into the hydrophobic membrane core (Figure 1.3b, c). As the concentration of QACs increases, the membrane core decreases in hydrophobicity and the phospholipids rearrange towards a stable hexagonal arrangement (Figure 1.3e, f). The formation of the QAC/phospholipid micelles causes bacterial cell lysis [16]. This appears to be a bactericidal effect.

#### 1.5.1.2 Alexidine

Alexidine (2-ethyl hexyl bisbiguanidine dihydrochloride) is a cationic antimicrobial agent belonging to the bisbiguanide family. As shown in Figure 1.4 by the two shaded outer circles, alexidine is a dimer with two active sites (i.e. two cationic groups) [52]. It has been used in the dental industry as a safe and effective disinfectant for decades and is currently being looked at as a possible antimicrobial agent for use in the field of ophthalmology [44]. I performed an Ovid medline search but was unable to find anything published on the ocular effect of alexidine.

Alexidine's mechanism of action against bacterial cells is very similar to that of BAK. Briefly, the positively charged cationic groups associate strongly with exposed anionic sites on the cell membrane and cell wall; they bind in particular to the head groups of the bacterial phospholipids. Binding of alexidine to the bacterial cell is stronger than the binding of BAK [16, 53]. Disruption and eventual lysis of the target

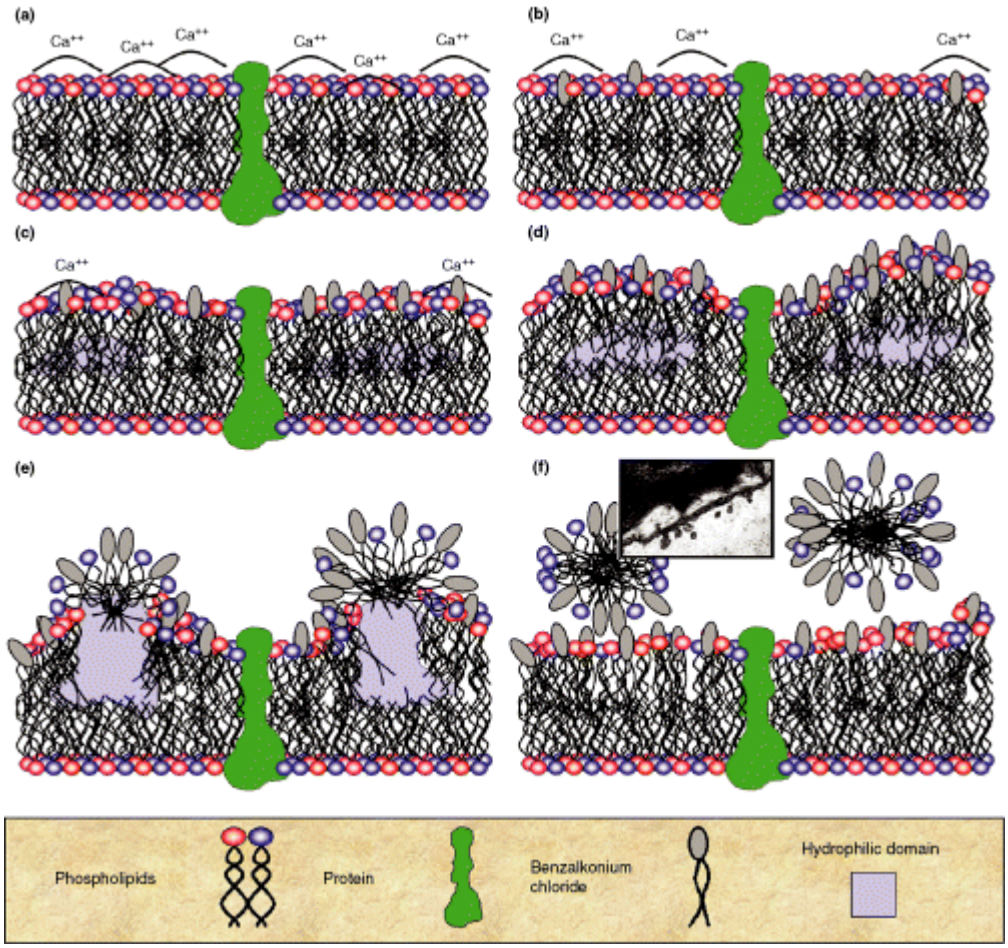


**Figure 1.3:** Cartoon showing the mechanism of action for quaternary ammonium biocides. The segments (a–f) show progressive adsorption of the quaternary headgroup to acidic phospholipids in the membrane with increasing QAC exposure/concentration. This leads to decreased fluidity of the bilayers and the creation of hydrophilic voids in the membrane. Protein function is perturbed with an eventual lysis of the cell, and solubilization of phospholipids and proteins into QAC/phospholipid micelles. Inset micrograph shows vesicle formation from outer membrane caused by QAC treatment [16].

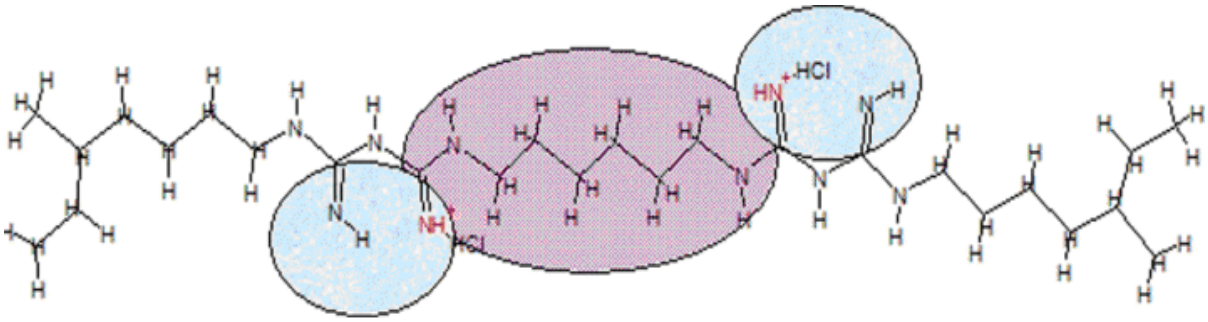


**Figure 1.4:** Chemical structure of the bisbiguanide alexidine. Cationic phospholipid binding sites are indicated by the two outer circles. Hydrophobic hexamethylene group indicated by the centre circle [16].

bacterial cell is brought about by the binding and disruption at these sites. A cartoon detailing the mechanism of action at the molecular level is outlined in Figure 1.5. It is evident from the cartoon that one of the major differences between alexidine and BAK is that hydrophobic regions of BAK become solubilized within the hydrophobic core of the cell membrane while those of alexidine do not [16]. The hydrophobic region of alexidine, represented by the inner shaded circle in Figure 1.4, is somewhat inflexible and is therefore incapable of folding sufficiently to interdigitate into the lipid bilayer [16]. Alexidine therefore bridges between pairs of adjacent phospholipid headgroups (Figure 1.5d). The end result is cellular leakage and bacterial cell death. This appears to be a bactericidal effect.

### 1.5.2 Tigecycline

Tigecycline is the first agent in a new class of antimicrobial agents, the glycylcyclines, which are semi-synthetic tetracyclines. As such tigecycline is structurally similar to the tetracycline family. Structural modifications of the four-ring carbocyclic structure characteristic of the tetracyclines resulted in tigecycline; specifically the t-butyglycylamido group was added at C-9 (Figure 1.6). The basic nitrogen of the glycyl unit is vital to the preservation of antibacterial activity [54]. Tigecycline is the only new antibiotic to be developed within the past 10 years to have a novel mechanism of action and broad-spectrum activity against bacterial organisms [43]. Tigecycline demonstrates broad-spectrum *in vitro* and *in vivo* activity against a wide spectrum of aerobic and anaerobic Gram-positive and Gram-negative organisms including resistant strains such as methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus*, and extended-

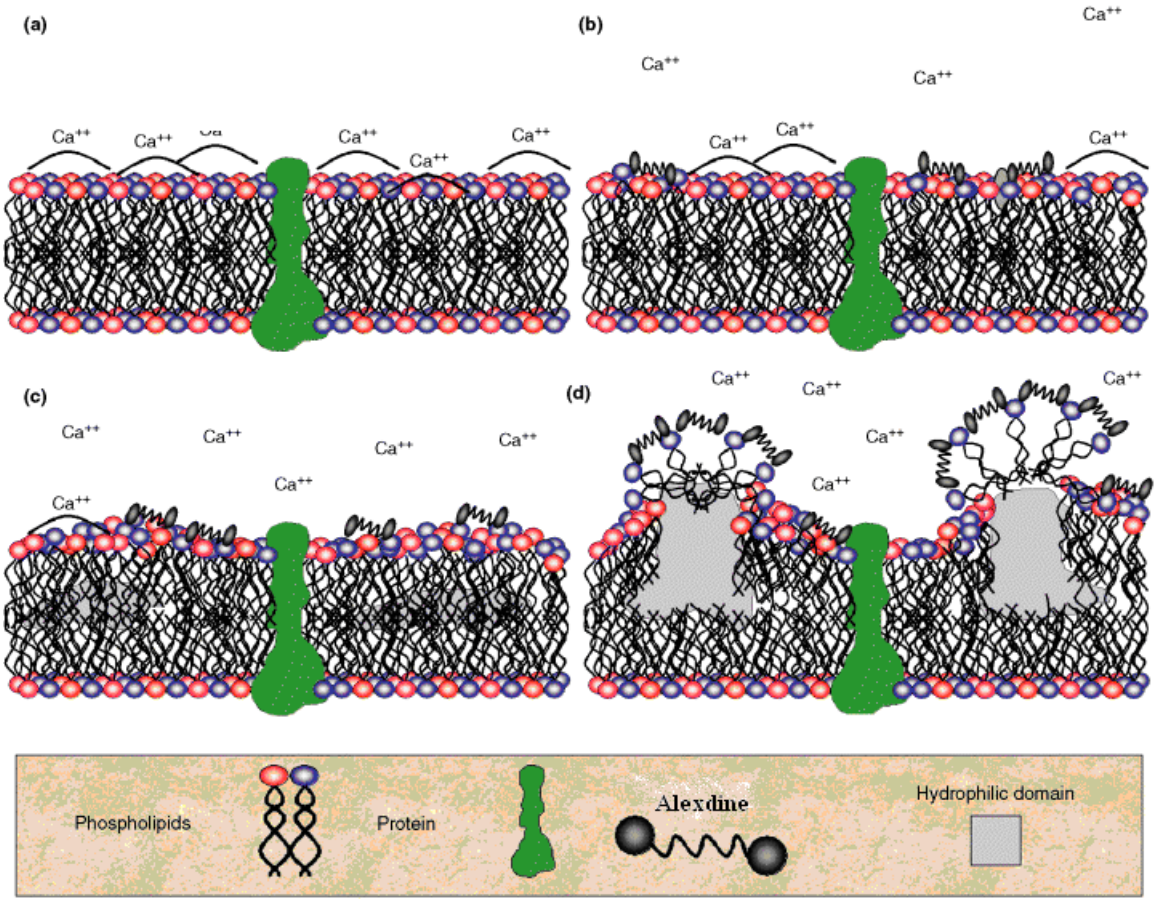


**Figure 1.5:** Diagramatic representation of the interaction of alexidine with the bacterial cytomplasmic membrane. Diagram shows progressive decreases in fluidity of the outer leaflet with increasing exposure to the bisbiguanide [16].

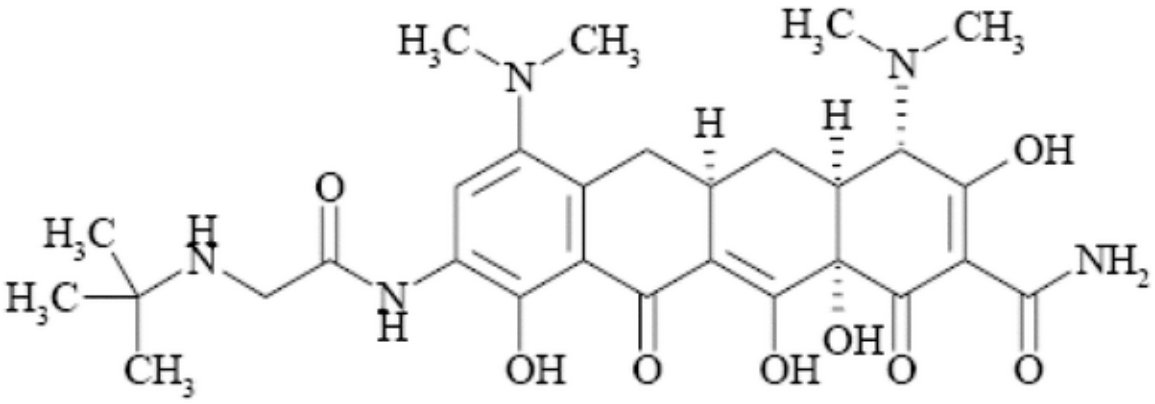

**Figure 1.6**: The molecular structure of tigecycline.

spectrum beta-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* [12, 54-57]. The United States Food and Drug Administration (FDA) approved tigecycline in June of 2005 for the treatment of complicated skin and skin structure infections and complicated intra-abdominal infections. Tigecycline is also approved for use in Canada.

#### 1.5.2.1 Mechanism of Action

Tigecycline is a bacteriostatic antimicrobial agent that inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit. The tetracyclines and glycylcyclines (i.e. tigecycline) share a binding site on the 30S ribosomal subunit, but the glycylcyclines interact directly with an additional site that the tetracyclines do not [58]. This additional binding allows tigecycline to bind five-fold more strongly to the ribosome than the tetracylines [43, 56]. Binding to the bacterial ribosome prevents translation by blocking the entry of amino-acyl transfer RNA into the ribosome and ultimately by preventing the incorporation of amino acids into the elongating peptide chain. The binding of tigecycline to the 30S ribosomal subunit is reversible, accounting for the bacteriostatic activity of tigecycline against susceptible organisms.

Tigecycline was developed in response to the growing resistance of Gram-positive and Gram-negative organisms to tetracycline [43]. The two major mechanisms of tetracycline resistance are ribosomal protection and active efflux of drug from the inside of the bacterial cell [12, 43, 56]. In studies thus far, it appears that tigecycline is able to evade these resistance mechanisms commonly affecting tetracyclines. It is believed that tigecycline's ability to overcome these two resistance mechanisms is due to steric hindrance created by the large substituent (t-butylglycylamido) at C-9 [54].

## 1.6 Determining Antimicrobial Susceptibility

One of the most important ways to prevent or reduce antimicrobial resistance is to treat infections with the appropriate antimicrobial agent at the appropriate concentration [11, 59, 60]. If used at an "appropriate" concentration, an antimicrobial agent should be able to prevent the growth of all susceptible and resistant bacterial cells within a cell population [8]. In a clinical setting, the clinical microbiology laboratory tests to determine the susceptibility of bacterial isolates from patient specimens to different antimicrobial agents. There are a number of types of susceptibility tests available, and the sensitivity and specificity of these tests can vary [61, 62]. In North America, an organism is called susceptible if its experimentally determined minimum inhibitory concentration (MIC) value for a particular antimicrobial agent meets or falls beneath the susceptibility breakpoints set forth by the Clinical Laboratory and Standards Institute (CLSI). In Europe susceptibility breakpoints are set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). To date, considerations in the setting of these breakpoints include the distribution pattern of MICs as well as the association between MIC and clinical efficacy [61, 63].

### 1.6.1 Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial agent required to inhibit bacterial growth of an inoculum containing $10^5$ colony forming units (CFUs) per milliliter (ml) [64]. Using CLSI susceptibility breakpoints, a bacterial strain can be characterized as susceptible, of intermediate resistance, or resistant to a particular antimicrobial agent on the basis of MIC values. There are currently several methods used to determine MIC values for

specific antimicrobial agent-bacterial organism combinations. These methods include but are not limited to the microbroth dilution method, the disc-diffusion method, the E-test method, and automated methods such as the VITEK system (BioMerieux, St. Laurent, QC). Standard protocols for all aforementioned tests are found in the CLSI and EUCAST guidelines [65, 66].

Minimum inhibitory concentration testing is a globally standardized and accepted test of susceptibility, however, there are several limitations involved with the use of MIC testing. One of the major limitations of using MIC values as a guideline for determining antibiotic susceptibility is that MIC values are experimentally determined using an organism density of $10^5$ CFU/ml. In 1942, Firsch and colleagues estimated that a bacterial burden of $10^{10}$ to $10^{12}$ CFUs was present during an acute infection in patients with pneumococcal pneumonia [67]. It seems reasonable that many more patients may be infected with greater numbers of bacterial organisms than those that are used in our current standardized MIC susceptibility testing (i.e. $10^5$ CFU/ml). Another important limitation of MIC testing is that it in no way indicates the possibility or extent of resistance selection. The mutant prevention concentration (MPC) was developed as a method to overcome the limitations of MIC testing.

### 1.6.2 Mutant Prevention Concentration

The mutant prevention concentration (MPC) is defined as the lowest concentration of antimicrobial agent required to inhibit the least susceptible bacterial cell in a bacterial inoculum of $\geq 10^9$ CFUs [8, 10, 68]. The term "mutant prevention concentration" was coined by Dong *et al* in 1999, following the recognition of a two-stage decline in CFUs when high-density bacterial inocula were exposed to varying

antimicrobial drug concentrations (Figure 1.7) [69]. Dong *et al* found that with increasing fluoroquinolone concentration there were two sharp decreases in the fractions of viable cells recovered when wild-type cells were used in experiments (in this case *Mycobacterium bovis* BCG and *Staphylococcus aureus*). The first decrease occurred at a fluoroquinolone concentration approximating the $MIC_{99}$ of the wild-type cells used. The second decline in recovered viable cells occurred at a higher fluoroquinolone concentration and followed a plateau region (i.e. a concentration range in which the recovery of viable cells decreased very gradually). Nucleotide sequence analysis of colonies recovered from the plateau region revealed mutations in the QRDR of GyrA (*Mycobacterium bovis* BCG) and ParC (*Staphylococcus aureus*). The authors concluded that the second large decline in viable cells occurred once the fluoroquinolone concentration was sufficient to block the growth of these first-step mutants; a concentration they termed the mutant prevention concentration [69].

The concept of the MPC is increasingly important because there are data to suggest that many patients may be infected with greater numbers of bacterial organisms than those that are used in our current standardized MIC susceptibility testing (i.e. $10^5$ CFUs), as described earlier [10, 67]. The MPC is experimentally determined using a bacterial burden that may more closely reflect the situation found in clinical infection (i.e $\geq 10^9$ CFUs). For fluoroquinolones, mutations are thought to occur at a rate of $1x10^{-7}$ to $1x10^{-9}$ [68]. Therefore, in order for a bacterial strain to grow in the presence of an antimicrobial agent present at the MPC concentration, it would require more than one mutation. The rationale for this is as follows: at a mutational frequency of $10^{-7}$, more than $10^{14}$ bacteria ($10^7$ x $10^7$) would be required to detect two concurrent resistance

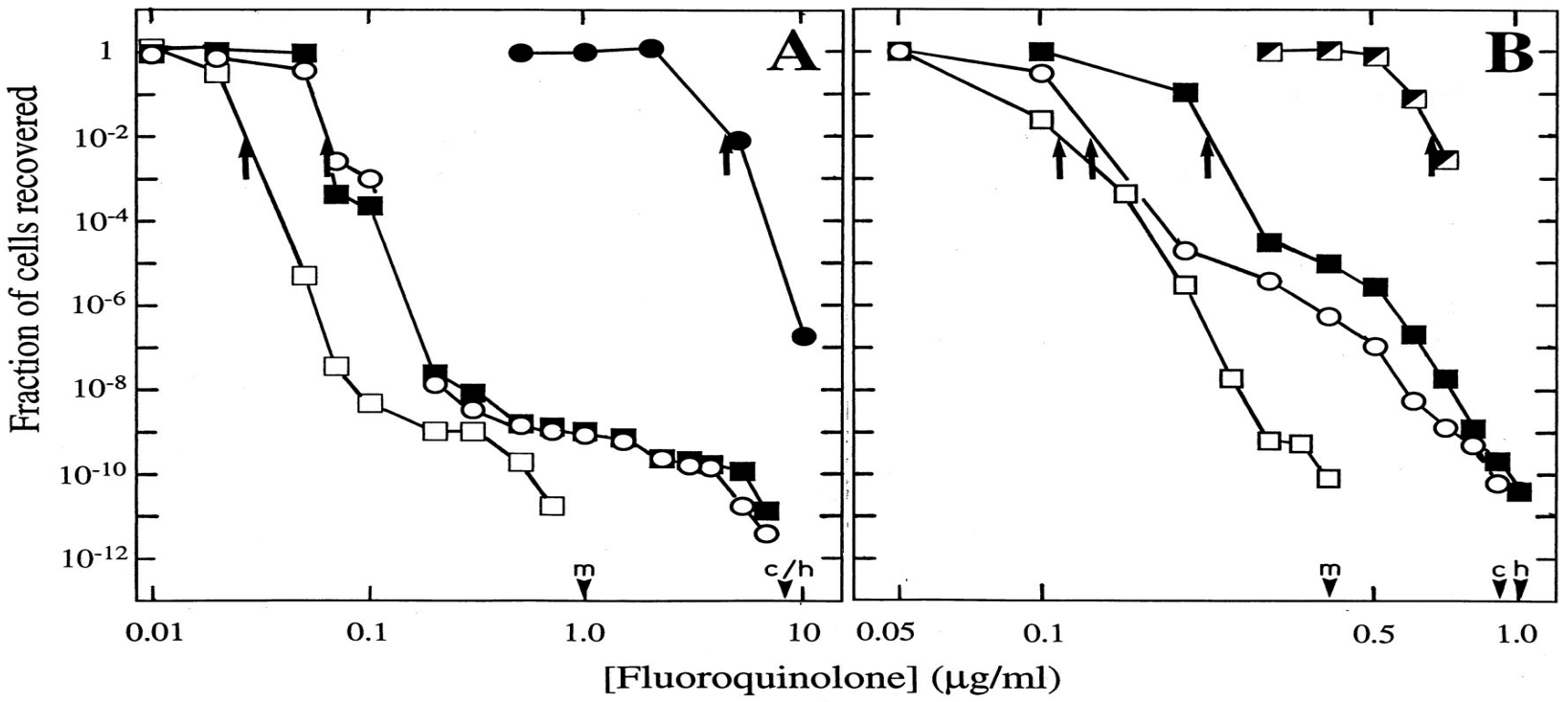


**Figure 1.7:** Effect of fluoroquinolone concentration on selection of resistant mutants. *M. bovis* BCG isolate KD1295 (A) and *S. aureus* MT5 (B) were plated on agar containing the indicated concentrations of PD161148, a C-8-methoxy compound (open squares), PD160793, a C-8-H derivative (filled squares), or ciprofloxacin (open circles). Panel A also shows the responses of the *M. bovis* BCG first-step mutant CX1 (3) to ciprofloxacin (filled circles); in panel B, half-filled squares show the responses of a first-step *parC* (Cipr) mutant of *S. aureus* (strain KD1806) to treatment with the indicated concentrations of PD160793. After incubation to allow growth, colonies were counted, and the fraction of the input number was determined. In the experiments shown, up to 1011 cells were applied to agar plates. The MIC99 for each compound is indicated by arrows. Small arrowheads on the abscissa indicate the MPC1010 for the C-8-methoxy compound (m), C-8-H compound (h), and ciprofloxacin (c) [69].

mutations. A bacterial load of $10^{14}$ is higher than that present during infectious diseases [8, 59].

Some authors have suggested that the MPC method of testing only applies to fluoroquinolone compounds [70]. Subsequently, numerous studies have experimentally measured MPC values against a wide variety of antimicrobial agents and bacterial pathogens [10]. It is important to remember that MPC defines the mutant prevention concentration and not the mutation prevention concentration. The MPC is used to determine the antimicrobial concentration required to block the growth of the least susceptible cell in the population and is independent of the mechanism of resistance.

#### 1.6.2.1 The Mutant Selection Window

The mutant-selection window (MSW) defines an antimicrobial concentration range in which the antimicrobial-resistant mutant subpopulation (present prior to treatment) is selectively amplified (Figure 1.8). The lower boundary of the MSW is the MIC, at which growth of all antibiotic-susceptible bacterial cells is inhibited. The upper boundary is defined by the MPC which blocks the growth of the least susceptible bacterial cell in the population. The MSW postulates that antimicrobial concentrations falling within the "window" may selectively amplify the resistant subpopulation present as part of the total bacterial burden [8, 10, 59]. We have previously demonstrated *in vitro* that when drug concentrations are in excess of the MPC, both susceptible and mutant organisms are inhibited [8]. When drug concentrations fall below the MIC, neither mutant nor susceptible cells are inhibited. For drug concentrations that fall within the MSW, susceptible cells are likely to be inhibited as the drug concentration is in excess of the MIC; however, mutant cells will not be inhibited as the drug concentration is below

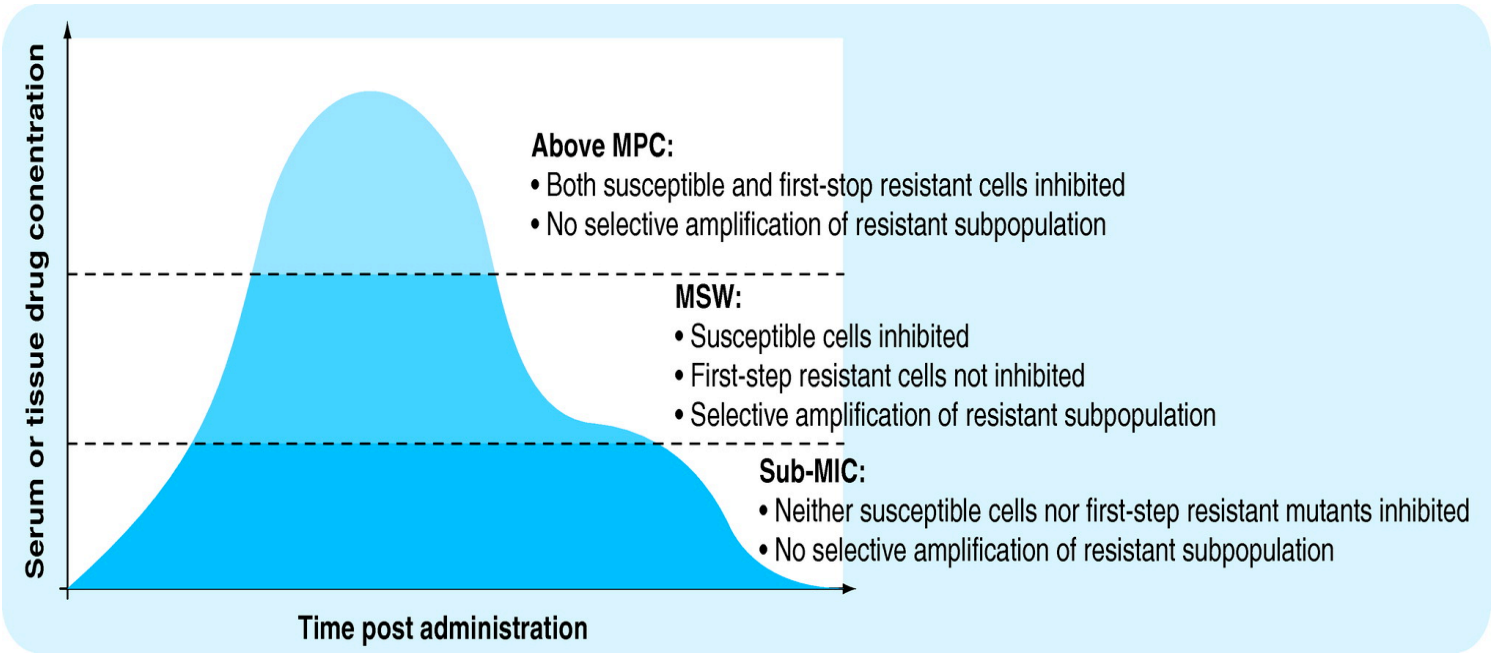


**Figure 1.8**: Mutant-selection window.
MIC: Minimum inhibitory concentration; MPC: Mutant-prevention concentration; MSW: Mutant-selection window [10].

the MPC. Therefore, although therapeutic drug concentrations used in clinical practice today lead to clinical cure, they may in fact be the same drug concentrations that are selectively amplifying the mutant subpopulation present in high-density bacterial burdens (Figure 1.9) [10]. Blondeau points out that "while not all resistance results in clinical failure, antimicrobial resistant pathogens increase the risk of drug failure" [71].

## 1.7 *In vitro* Growth Dynamics

### 1.7.1 Time-Kill Curves

Bacterial susceptibility testing methods such as MIC and MPC are useful tools for determining drug-concentrations required to inhibit bacterial growth *in vitro*, however, these tests reveal nothing about the killing potential of an antimicrobial agent [71]. Time-kill experiments are performed to measure the $\log_{10}$-reduction and percent kill of viable bacterial cells when exposed to different antimicrobial agents. One limitation of time-kill experiments is that these studies have a constant amount of drug (i.e. there is no drug elimination over time as there is in humans) [8]. In spite of this limitation, these experiments remain valuable tools, as the bacterial load, antimicrobial concentration, and duration of the assay can all be controlled as desired.

## 1.8 Summary

Antimicrobial resistance is a growing concern worldwide. If bacterial pathogens continue to develop resistance to antimicrobial agents at the current rate, we will inevitably end up in a situation that resembles the pre-antibiotic era in which no therapeutically-useful antibiotics are available. In order to prevent an era where people will once again die from simple bacterial infections, it is critical that as a society we take measures to ensure the responsible use of antimicrobial agents. One of the most

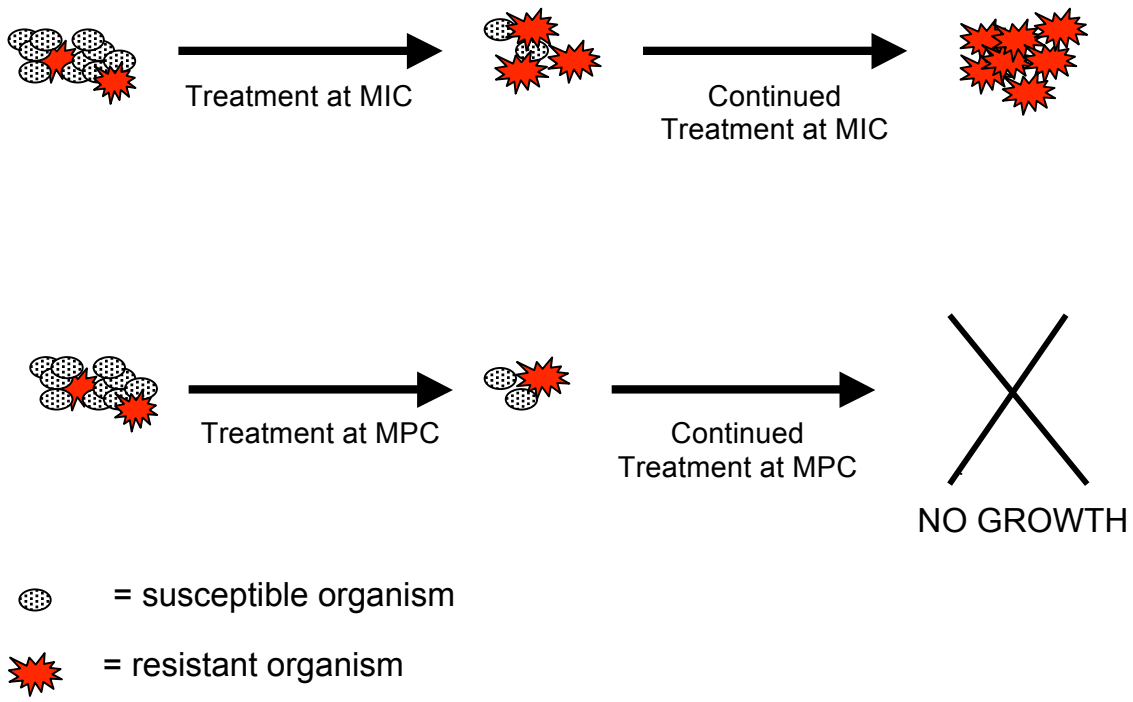


**Figure 1.9:** Cartoon illustrating potential treatment outcomes using both MIC and MPC drug concentrations.

important means of reducing the selective pressure that helps resistant organisms emerge is using the appropriate drug at the appropriate dosage and for the appropriate duration [14].

The current susceptibility breakpoints, as outlined in the CLSI or EUCAST guidelines, may actually be providing physicians with dosing information that is ultimately leading to increased antibacterial resistance. In susceptibility testing, there is a need for methods that can give an indication of the possibility or extent of resistance selection; the MPC method is one such method. Use of the MPC method in clinical laboratories may be one potential answer to the global pandemic of antimicrobial resistance.

**1.9 Objectives**

Four main objectives were established for this graduate research project. The first objective was to develop new experimental methods for determining MPC values. The second objective was to determine if MPC testing of tigecycline against *S. pneumoniae* is impacted by media, specifically media containing blood. The third objective was to determine whether the flouroquinolones gatifloxacin and moxifloxacin, are more active (lower MIC values) against bacterial pathogens in the presence of BAK. The fourth and final objective was to conduct susceptibility testing of Alexidine using MIC testing, MPC testing, and time-kill curves against Gram-positive and Gram-negative pathogens.

## 2.0 MATERIALS AND METHODS

### 2.1 Standard Laboratory Methods

#### 2.1.1 Isolate Collection and Identification

Clinical isolates of *Staphylococcus aureus* (MRSA, MSSA, CNS), *Streptococcus pneumoniae*, *Escherichia coli*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* were collected (2000-2007) from the Clinical Microbiology Laboratory, Royal University Hospital, Saskatoon, SK. Isolates were from patients with various infections and were identified by Vitek (BioMerieux, St. Laurent, QC) and other reference procedures as summarized in the Manual of Clinical Microbiology [64]. Methicillin-resistance was detected by inoculation to and incubation on a Mueller-Hinton oxacillin screen plate and then confirmed using an "in-house" polymerase chain reaction assay to detect the *mecA* gene.

No pre-selection criteria were used that would favor the inclusion or exclusion of organisms with specific susceptibilities to the various antimicrobial agents tested. Care was taken to ensure that duplicate isolates from the same patient were excluded.

American Type Culture Collection (ATCC) strains for Methicillin-susceptible *Staphylococcus aureus* (MSSA) (ATCC 29213 and ATCC 25923), Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300), *S. pneumoniae* (ATCC 49619), *H. influenzae* (ATCC 49427), *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853) were obtained from the American Type Cultures Collection (Rocville, MD). ATCC strains were used as controls for susceptibility testing and were tested each time a susceptibility test was performed.

### 2.1.2 Storage of the Bacterial Isolates

*Staphylococcus* spp., *P. aeruginosa*, and *E. coli* isolates were streaked for isolated colonies on Tryptic Soy Agar (TSA) plates (in house) and incubated in $O_2$ at 35 to 37°C for 18 to 24 hours. *S. pneumoniae* isolates were streaked for isolated colonies on TSA plates containing 5% sheep red blood cells (SRBCs) (Oxoid, Ryegate, MT) and incubated in $CO_2$ at 35 to 37°C for 18 to 24 hours. *H. influenzae* isolates were streaked for isolated colonies on Chocolate agar plates (PML Microbiologicals, Winnipeg, MB) and incubated in $CO_2$ at 35 to 37°C for approximately 24 hours [64]. Following incubation, colonies were inoculated using a sterile wooden applicator stick into 1.2 ml Corning cryovials containing 0.5 ml of skim milk. The vials were stored at -70°C.

## 2.2 Susceptibility Testing

The antimicrobial agents used for *in vitro* experiments were obtained either in powdered form or in solution. Those antimicrobials obtained in powdered form were diluted in accordance to the manufacturer's recommendations. Sources of antimicrobial agents were as follows:

Alexidine (solution) – Bausch and Lomb Pharmaceuticals Inc., Rochester, NY, U.S.A.;

Benzalkonium chloride (BAK) (solution) – Allergan Inc., Irvine, CA, U.S.A.;

Gatifloxacin (powder) – Bristol Myers Squibb, Montreal, QC, Canada;

Gemifloxacin (powder) – Glaxo Smith Kline Pharmaceuticals, Collegeville, PA, U.S.A.;

Oscient Pharmaceuticals, Waltham, MA, U.S.A.;

Levofloxacin (solution) – Janssen Ortho, Toronto, ON, Canada;

Moxifloxacin (powder) – Bayer Pharmaceutical, Toronto, ON, Canada;

Tigecycline (powder) – Wyeth Pharmaceuticals, Collegeville, PA, U.S.A;

Vigamox (commercial solution) – Alcon, Fort Worth, TX, U.S.A.;

Zymar (commercial solution) – Allergan Inc., Irvine, CA, U.S.A.

### 2.2.1 Broth Microdilution

The broth microdilution method was used to determine minimum inhibitory concentration (MIC) values for all organisms in accordance with the Clinical Laboratory and Standards Institute (CLSI) guidelines [65]. To prepare 96-well microtitre panels (Sarstedt, Newton, NC) used for broth microdilution testing, 100 μl of appropriate broth was added to each well of columns 2 to 12. Mueller-Hinton Broth (MHB) (Becton Dickinson, Sparks, MD) was used for *Staphylococcus* spp., *E. coli*, and *P. aeruginosa*; Todd-Hewitt Broth (THB) (Becton Dickinson, Sparks, MD) was used for *S. pneumoniae*; and Pluronic Inoculum water for *H. influenzae*. Antimicrobial agent was serially diluted down the panel with the wells of column 1 containing the highest concentration of drug and the wells of column 11 the lowest concentration of drug. The wells of column 12 were used as a growth control and, therefore, did not receive any drug.

Organisms were subcultured onto TSA + 5% SRBC plates and incubated under appropriate conditions for 18 to 24 hours at 35 to 37°C: *S. pneumoniae* and *H. influenzae* in 5% $CO_2$, *Staphylococcus* spp. *E. coli*, and *P. aeruginosa* in $O_2$. Following incubation, each organism was standardized to a 0.5 McFarland (~1.0 X $10^8$ CFU/ml) using a colorimeter. The bacterial suspensions were then diluted 1/100 with the appropriate broth medium (as above except *H. influenzae* is diluted with Brain Heart Infusion plus 5% fildes (Becton Dickinson, Sparks, MD)) to give a bacterial load of approximately 1.0 X $10^6$ CFU/ml. One hundred μl of the bacterial suspension was then added to each well in the microtitre panel resulting in a final bacterial concentration of approximately 1.0 x

$10^5$ CFU/ml. The microtitre panels were then incubated under the appropriate conditions (as above) for 18 to 24 hours at 35 to 37°C. Following incubation, the lowest drug concentration at which there was no visible growth of organism was recorded as the MIC.

A sample of each bacterial suspension was plated onto fresh TSA + 5% SRBC plates and incubated in ambient air at 35 to 37°C for 18 to 24 hours to ensure the purity of the bacterial suspensions. The wells in column 12 (growth control) were also examined to ensure organism viability. The appropriate ATCC strain(s) was/were used in each assay as a control to confirm the validity of the results based on the current CLSI breakpoints for each ATCC strain.

**2.3 Mutant Prevention Concentration (MPC)**

Dong *et al*. first defined the MPC concept and initial MPC method in 1999 [69]. The laboratory of Dr. J. Blondeau (Royal University Hospital, Saskatoon, SK) has since described MPC testing protocols for the following organisms: *S. pneumoniae [68]*, *S. aureus [72]*, *Staphylococcus intermedius*, *E. coli [73, 74]*, *P. aeruginosa [74, 75]*, *H. influenzae [76]*, *Citrobacter freundii [74]*, *Actinobacillus pleuropneumoniae*, *Enterobacter cloacae [74]*, *Klebsiella pneumoniae [74]*, *Pasteurella multocida*, *Histophilus somni*, and *Mannheimia haemolytica [77]*.

**2.3.1 Inoculum Preparation**

Preparation of the bacterial suspension required for MPC testing is tedious and time-consuming and different protocols are required for different bacterial organisms. The protocol is highly dependent upon the fastidiousness of the organism; the more fastidious an organism the more involved the protocol. The challenge in MPC testing is to generate bacterial suspensions of $\geq 10^9$ CFUs. In order to achieve such high bacterial

densities, confluent bacterial growth from several agar plates is transferred into the appropriate broth medium and incubated for the appropriate time at the appropriate conditions. There are currently two methods available to determine the MPC value of a particular microorganism to a particular antimicrobial compound: the traditional agar dilution method and the novel microbroth dilution method. Both methods were developed in the laboratory of Dr. J. Blondeau, Royal University Hospital, Saskatoon, SK.

### 2.3.2 Traditional MPC testing: Agar Dilution Method

The traditional MPC testing method is the agar dilution method. Bacterial isolates were streaked from thawed skim milk onto TSA + 5% SRBC plates using a sterile wooden stick and incubated under ambient conditions at 35 to 37°C for 18 to 24 hours. Following incubation, growth from the original plate was transferred to fresh TSA + 5% SRBC plates using a sterile swab. The entire surface of the fresh plates was inoculated to ensure confluent bacterial growth. The number of plates inoculated was dependent on the fastidiousness of the organism; *S. pneumoniae* was inoculated onto 8 plates and *Staphylococcus spp.* onto 3 plates. Once the fresh TSA + 5% SRBC plates were inoculated they were incubated under the appropriate conditions for a further 18 to 24 hours at 35 to 37°C; *S. pneumoniae* was incubated in 5% $CO_2$ and *Staphylococcus* spp. in $O_2$.

After overnight incubation, the plates containing confluent bacterial growth were transferred, using a sterile swab, into a defined volume of appropriate broth; *S. pneumoniae* into 500 ml of THB and *Staphylococcus* spp. into 100 ml of MHB. Following the transfer of organism into the appropriate broth, another overnight incubation of 18 to 24 hours was required for the proliferation of the bacterial cells. *S.*

*pneumoniae* is a fastidious organism and requires a centrifugation step in order to achieve the large bacterial densities required for MPC testing. To concentrate the bacterial load of *S. pneumoniae*, cultures were centrifuged at 8,000 rpm for 20 minutes at 4°C and then re-suspended in 3 ml of fresh THB. To ensure that the cellular concentration of each isolate was of sufficient density ($\geq 10^9$ CFUs), an absorbance reading was taken using a spectrophotometer. For *S. pneumoniae* an absorbance reading of ≥0.3 at a wavelength of 600 nm was used to estimate a cellular density of $\geq 10^9$ CFU/ml and for *Staphylococcus* spp. an absorbance reading of ≥1.0 at a wavelength of 600 nm was used to estimate a cellular density of $\geq 10^{10}$ CFU/ml. Viable counts were performed on the high-density bacterial cultures to ensure that a concentration of $\geq 10^9$ CFU/ml existed. A purity plate was prepared using a sterile stick to streak out the high-density bacterial suspension onto a TSA + 5% SRBC plate. The purity plates were incubated under the appropriate conditions ($O_2$ for *Staphylococcus* spp. and $CO_2$ for *S. pneumoniae*) for 18 to 24 hours at 35 to 37°C and examined for contaminants.

For each experiment, agar plates were prepared containing doubling dilutions of antimicrobial agent. In most cases seven plates were prepared for each isolate, each with a different concentration of antimicrobial agent. The range of drug concentrations tested included the measured MIC value of each isolate, one doubling dilution below the MIC value, and a minimum of five doubling dilutions above the MIC value. To create the agar dilution plates, the defined amount of antimicrobial agent was added to liquid TSA + 5% SRBC agar was also added to agar on which *S. pneumoniae* was to be grown. The agar was then poured into plastic petri plates (Fisher Scientific, USA) and allowed to solidify

overnight. Plates were kept at room temperature and used within 2 to 3 days of their preparation.

After taking absorbance readings and making certain that the bacterial cultures had cellular densities of $\geq 10^9$ CFU/ml, aliquots were applied to the prepared TSA + 5% SRBC plates containing differing concentrations of the antimicrobial agent of interest. For *S. pneumoniae*, aliquots of 200 μl were applied to the drug plates and for *Staphylococcus* spp. aliquots of 100 μl were applied to the drug plates. An appropriate and fully susceptible ATCC strain was included in each experiment: ATCC 49619 for *S. pneumoniae* and ATCC 29213 or ATCC 25923 for *Staphylococcus* spp. Following inoculation, plates were incubated under the appropriate conditions (ambient air for *Staphylococcus* spp. and 5% $CO_2$ for *S. pneumoniae*) for 18 to 24 hours at 35 to 37°C and then screened for growth. The plates were then incubated for a further 18 to 24 hours and re-screened for growth. The MPC was recorded as the lowest concentration of antimicrobial agent that allowed no visible bacterial growth.

In certain cases, a faint haze caused by the high-density inoculum was seen on plates. This faint haze rendered it difficult to interpret whether these plates were positive for growth; additional steps were therefore used to determine accurate MPC values. Potential growth was collected using a sterile swab and was transferred to a prepared TSA + 5% SRBC plate containing the same antimicrobial concentration as the plate in question. These fresh drug plates inoculated with potentially viable cells were then incubated for 18 to 24 hours in ambient air (5% $CO_2$ for *S. pneumoniae*) at 35 to 37°C and screened for growth. The MPC was recorded as the lowest drug concentration preventing visible growth.

### 2.3.3 Modified MPC testing: Microbroth Dilution Method

I developed a novel method for MPC testing: the modified microbroth dilution method [78]. A 96-well microtitre panel is used in this method instead of agar plates containing doubling dilutions of antimicrobial agent. The first step in this method is to generate a bacterial suspension that has a cellular density of $\geq 10^9$ CFU/ml. The protocol for generating such a high-density inoculum is the same as that used in the traditional MPC method. Bacterial isolates were streaked from thawed skim milk onto TSA + 5% SRBC plates using a sterile wooden stick and incubated under ambient conditions at 35 to 37°C for 18 to 24 hours. Following incubation, growth from the original plate was transferred to fresh TSA + 5% SRBC plates using a sterile swab. The entire surface of the fresh plates was inoculated to ensure confluent bacterial growth. The number of plates inoculated was dependent on the fastidiousness of the organism; *S. pneumoniae* was inoculated onto 8 plates and *Staphylococcus spp.* onto 3 plates. Once the fresh TSA + 5% SRBC plates were inoculated they were incubated under the appropriate conditions for a further 18 to 24 hours at 35 to 37°C; *S. pneumoniae* was incubated in 5% $CO_2$ and *Staphylococcus* spp. in $O_2$.

After overnight incubation, the plates containing confluent bacterial growth were transferred, using a sterile swab, into 10 ml of broth; *S. pneumoniae* into THB and *Staphylococcus* spp. into MHB. Following the transfer of organism into broth, an incubation period of 2 hours at 35 to 37°C under ambient conditions was required for the proliferation of the bacterial cells. At this point viable counts were performed on the high-density bacterial cultures to ensure that a concentration of $\geq 10^9$ CFU/ml was present and each isolate was streaked onto a TSA + 5% SRBC plate using a sterile wooden

applicator stick. The TSA + 5% SRBC plate was incubated in ambient air for 18 to 24 hours at 35 to 37°C and then screened to ensure each isolate was pure. An appropriate and fully susceptible ATCC strain was included in each experiment: ATCC 49619 for *S. pneumoniae* and ATCC 29213 or ATCC 25923 for *Staphylococcus* spp.

One hundred μl of each high-density bacterial suspension was added to all wells in a single row in a 96-well microtitre panel prepared with doubling dilutions of antibiotic. To prepare 96-well microtitre panels, 100 μl of the appropriate broth (THB for *S. pneumoniae* and MHB for *Staphylococcus* spp.) was added to each well in columns 2 to 12. Antimicrobial agent was serially diluted down the 96-well microtitrepanel with the wells of column 1 containing the highest concentration of drug and the wells of column 11 the lowest concentration of drug. The wells of column 12 were used as a growth control and therefore did not receive any drug. The final bacterial concentration in each well is diluted 1/10 from the concentration of the initial stock tube. For example, if the concentration of the initial bacterial suspension was $1.0 \times 10^{10}$ CFU/ml, the final bacterial burden would be approximately $1.0 \times 10^{9}$ CFUs following the addition of 100 μl of bacterial suspension to each well. After adding 100 μl of each bacterial suspension, the microtitre panels were incubated in ambient air (5% $CO_2$ for *S. pneumoniae*) for 18 to 24 hours at 35 to 37°C.

Following incubation the microtitre panels are screened for growth and the lowest drug concentration showing no visible growth is the MPC. In some instances the wells were too cloudy to read due to the very high cellular concentration (debris) and additional steps were required to determine accurate MPC values. A sterile wooden applicator stick was placed into each well that was difficult to read and was subsequently used to streak a

TSA + 5% SRBC plate containing the corresponding concentration of antimicrobial agent. In order for this additional step to be completed, agar dilution plates must be prepared as detailed in the traditional MPC method. The TSA + 5% SRBC plates are incubated in ambient air for 18 to 24 hours at 35 to 37°C.

### 2.3.4 Viable Counts

When completing traditional MPC testing, viable counts were performed, after measuring absorbance values, on isolates with the highest and lowest absorbance readings. The initial bacterial suspensions were diluted to $10^{-7}$, $10^{-8}$, and $10^{-9}$ and 100 μl of each dilution were plated onto triplicate TSA + 5% SRBC plates. The plates were incubated in ambient air for 24 hours at 35 to 37°C. Following incubation, colony counts were performed on each plate and calculations completed to determine the bacterial concentration in CFU/ml. When using the modified microbroth dilution MPC testing method, viable counts were performed on each isolate using the same method.

### 2.3.5 E-test

E-test was used as a potential method of determining MPC values for *S. aureus* isolates. A bacterial suspension with a cellular density of $\geq 10^{10}$ CFU/ml is generated by streaking bacterial isolates from thawed skim milk onto TSA + 5% SRBC plates using a sterile wooden stick and incubated under ambient conditions at 35 to 37°C for 18 to 24 hours. Following incubation, growth from the original plate was transferred to 3 fresh TSA + 5% SRBC plates using a sterile swab. The entire surface of the fresh plates was inoculated to ensure confluent bacterial growth. Once the fresh TSA + 5% SRBC plates were inoculated they were incubated in ambient air at 35 to 37°C for a further 18 to 24 hours.

After overnight incubation, the plates containing confluent bacterial growth were transferred, using a sterile swab, into 10 ml of MHB and incubated for 2 hours in ambient air at 35 to 37°C. At this point viable counts were performed on the high-density bacterial cultures to ensure that a concentration of $\geq 10^{10}$ CFU/ml was present and each isolate was streaked onto a TSA + 5% SRBC plate using a sterile wooden applicator stick. The TSA + 5% SRBC plate was incubated in ambient air for 18 to 24 hours at 35 to 37°C and then screened to ensure each isolate was pure. A fully susceptible *S. aureus* control strain (ATCC 29213) was included in each experiment.

One hundred μl of each high-density bacterial suspension was added to MH agar plates and spread using a sterile cell spreader (Fisher Scientific, U.S.A.). E-test strips (Ab Biodisk, U.S.A.) were then added to the centre of the inoculated MH agar plate using sterile forceps and incubated in ambient air for 18 to 24 hours at 35 to 37°C. The plates were read as described by the E-test manufacturer and the lowest concentration inhibiting growth was taken as the MPC.

**2.4 Development of New Media for MPC Testing of Tigecycline**

The laboratory of Dr. J. Blondeau (Royal University Hospital, Saskatoon, SK) developed a novel formulation of Todd-Hewitt Broth for MPC testing of the antimicrobial agent tigecycline: solidified Todd-Hewitt Broth (sTHB). To our knowledge we are the first to use sTHB for susceptibility testing [79-81]. Solidified Todd-Hewitt Broth was prepared by adding 1.5% granulated agar (Becton Dickinson, Sparks, MD) to THB powder, dissolved in the appropriate volume of water (as described by the manufacturer), and autoclaving for 15 minutes at 121°C. Following removal from the autoclave, the liquid agar was cooled to 55°C in a water bath. Antimicrobial agent

was added to the liquid agar, stirred and then approximately 20 ml was poured into sterile petri plates and allowed to solidify. ATCC strains were inoculated to these plates, incubated as described, and then screened to ensure organism growth.

**2.5 Time - Kill Experiments**

Bacterial isolates were streaked from thawed skim milk onto TSA + 5% SRBC plates using a sterile wooden stick and incubated under ambient conditions at 35 to 37°C for 18 to 24 hours. Following incubation, growth from the original plate was transferred to fresh TSA + 5% SRBC plates using a sterile swab. The entire surface of the fresh plates was inoculated to ensure confluent bacterial growth; *S. pneumoniae* was inoculated onto 6 plates while *Staphylococcus* spp. and *P. aeruginosa* were inoculated onto one plate. Once the fresh TSA + 5% SRBC plates were inoculated they were incubated under the appropriate conditions for a further 18 to 24 hours at 35 to 37°C: *Staphylococcus* spp. and *P. aeruginosa* were incubated in ambient air and *S. pneumoniae* was incubated in 5% $CO_2$.

Following incubation, bacterial growth from the plates was transferred with a sterile swab to 10 ml of broth and incubated for 2 hours under the appropriate conditions. For *Staphylococcus* spp. and *P. aeruginosa*, half a plate of bacterial growth was transferred to MHB and incubated in ambient air. Six full plates of bacterial growth were transferred to THB and incubated in 5% $CO_2$ for *S. pneumoniae*. A viable count of the bacterial suspension was taken and corresponded to a bacterial density (CFU/ml) of $10^9$, $10^9$, and $10^7$ for *Staphylococcus* spp., *P. aeruginosa*, and S. *pneumoniae* respectively. To achieve an inoculum concentration of $10^5$ CFU/ml, the 10 ml suspensions were serially diluted; 700 μl was removed and added to 7 ml of fresh broth. At this point antimicrobial

agent was added to the $10^5$ CFU/ml dilutions at concentrations corresponding to the MIC, 2 x MIC, and 4 x MIC. The $10^5$ CFU/ml dilutions, containing antimicrobial agent, were vortexed and incubated under the appropriate conditions as described for 180 minutes. This was done in triplicate.

One hundred μl aliquots of the $10^5$ CFU/ml dilutions were taken at 0, 15, 30, 45, 60, 75, 90, 120, and 180 minutes after the addition of antimicrobial agent. The aliquots were diluted 1/10 in the appropriate broth and applied to TSA + 5% SRBC plates in triplicate and incubated at 35 to 37°C for 18 to 24 hours under the appropriate conditions. Following incubation, the number of colonies on each plate was recorded and the $\log_{10}$-reduction (percent kill) of viable cells was calculated. The aliquots were diluted 1/10 so that viable counts had a countable number of colonies (20 – 200). For some experiments aliquots were taken at 0, 0.5, 1, 2, 3, 4, 5, 10, and 15 minutes after the addition of antimicrobial agent and for other experiments at 0, 5, 10, 15, 20, 25, 30, 60, 120, and 180 minutes after the addition of antimicrobial agent.

## 3.0 RESULTS

### 3.1 Methods to Simplify MPC Testing

#### 3.1.1 Traditional MPC Testing

The traditional MPC testing method is the agar dilution method. Mutant prevention concentration values were experimentally determined, using the agar dilution method, for *Streptococcus pneumoniae* and Methicillin-susceptible *Staphylococcus aureus* (MSSA) ATCC control strains (ATCC 49619 and ATCC 29213 respectively) against gatifloxacin and moxifloxacin. The traditional MPC values for *S. pneumoniae* (ATCC 49619) against gatifloxacin and moxifloxacin were both 0.5 μg/ml (Table 3.1.1.1). The traditional MPC values for MSSA (ATCC 29213) against gatifloxacin and moxifloxacin were 8 μg/ml and 4 μg/ml respectively (Table 3.1.1.1).

#### 3.1.2 Modified MPC Testing

The modified MPC testing method is the microbroth dilution method. We developed this novel method in our laboratory and to our knowledge we are the only lab to use this method [78]. Mutant prevention concentration values were experimentally determined, using the microbroth dilution MPC method, for *S. pneumoniae* (ATCC 49619) and MSSA (ATCC 29213) control strains against gatifloxacin and moxifloxacin. The microbroth dilution MPC values for *S. pneumoniae* (ATCC 49619) against gatifloxacin and moxifloxacin were both 0.5 μg/ml (Table 3.1.1.1). The microbroth dilution MPC values for MSSA (ATCC 29213) against gatifloxacin and moxifloxacin were ≥8 μg/ml and >2 μg/ml respectively (Table 3.1.1.1). Comparison of the MPC values generated by the traditional- and microbroth dilution- MPC methods provided consistent results (Table 3.1.1.1), revealing the novel microbroth dilution MPC method as

| | MPC values (µg/ml) | | | |
|---|---|---|---|---|
| | ***S. pneumoniae* (ATCC 49619)** | | **MSSA (ATCC 29213)** | |
| **Drug** | **Traditional Method[a]** | **Microbroth Dilution Method** | **Traditional Method[a]** | **Microbroth Dilution Method** |
| **Gatifloxacin** | 0.5 | 0.5 | 8 | ≥8 |
| **Moxifloxacin** | 0.5 | 0.5 | 4 | >2 |

**Table 3.1.1.1: Comparison of the *in vitro* MPC values (µg/ml) generated using the traditional MPC method[a] and the microbroth dilution MPC method.**

[a] Agar Dilution Method

MPC = Mutant Prevention Concentration; MSSA = Methicillin-susceptible *Staphylococcus aureus*

a reliable method for determining MPC values. It is important to mention that when using the microbroth dilution MPC method for the testing of *S. pneumoniae*, the highest bacterial density achieved was $10^7$ CFU/ml because of the fastidiousness of *S. pneumoniae*. Regardless of the lower bacterial density, results were still consistent with those generated with higher bacterial densities (using the traditional MPC method).

#### 3.1.2.1 Inhibitory Values at Different Bacterial Concentrations

Antimicrobial susceptibility testing was performed using the microbroth dilution MPC method with bacterial concentrations ranging from $10^1$ to $10^9$ CFU/ml ($10^1$ to $10^7$ CFU/ml for *S. pneumoniae*) for *S. pneumoniae* (ATCC 49619) and MSSA (ATCC 29213) against gatifloxacin and moxifloxacin. The results of these experiments are summarized in Table 3.1.2.1.1. It is evident that as the bacterial burden increases so does the antimicrobial concentration required to inhibit growth. For MSSA (ATCC 29213), the largest increase in antimicrobial inhibitory concentration occurs when the bacterial burden increases from $10^6$ to $10^7$ CFU/ml; from 0.125 μg/ml to 4 μg/ml for gatifloxacin and from 0.5 μg/ml to >2 μg/ml for moxifloxacin. For *S. pneumoniae* (ATCC 49619), the antimicrobial inhibitory concentration increases steadily with increasing bacterial densities, with no large increase seen between any two consecutive bacterial concentrations.

The MIC and MPC values, as determined by the microbroth dilution MPC method, were compared with MIC and MPC values determined by the traditional MIC and MPC methods. The traditional MIC method was the microbroth dilution method with $10^5$ CFU/ml being achieved using a 0.5 McFarland standard. The comparison of these values is seen in Table 3.1.2.1.2 and also visually in Figures 3.1.2.1.1 and 3.1.2.1.2.

| Bacterial Concentration (CFU/ml) | Inhibitory Concentrations (µg/ml) | | | |
|---|---|---|---|---|
| | Gatifloxacin | | Moxifloxacin | |
| | ***S. pneumoniae* (ATCC 49619)** | **MSSA (ATCC 29213)** | ***S. pneumoniae* (ATCC 49619)** | **MSSA (ATCC 29213)** |
| **$10^1$** | 0.125 | 0.063 | 0.063 | 0.016 |
| **$10^2$** | 0.125 | 0.063 | 0.063 | 0.016 |
| **$10^3$** | 0.125 | 0.063 | 0.125 | 0.031 |
| **$10^4$** | 0.125 | 0.063 | 0.125 | 0.031 |
| **$10^5$** | 0.25 | 0.125 | 0.125 | 0.031 |
| **$10^6$** | 0.25 | 0.125 | 0.25 | 0.5 |
| **$10^7$** | 0.5 | 4 | 0.5 | >2 |
| **$10^8$** | | ≥8 | | >2 |
| **$10^9$** | | ≥8 | | >2 |

**Table 3.1.2.1.1: Inhibitory concentrations (µg/ml) for *S. pneumoniae* (ATCC 49619) and MSSA (ATCC 29213) against gatifloxacin and moxifloxacin at different bacterial concentrations.**

MSSA = Methicillin-susceptible *Staphylococcus aureus*

### 3.1.3 E-test

The E-test method is commonly used in clinical laboratories as a fast and simple way of determining MIC values; we looked at the E-test as a possible method for determining MPC values. E-test strips have a calibrated gradient of antimicrobial agent and when placed on agar plates with standardized concentrations of bacteria, the inhibitory antimicrobial concentration can be determined by establishing at what antimicrobial concentration bacterial colonies cross the calibrated strip. Traditionally E-test strips are used to determine MIC values, and as such a standardized bacterial inoculum of $10^5$ CFUs is applied confluently to the agar plates. We placed gatifloxacin E-test strips onto plates containing confluent growth of $\geq 10^9$ CFUs in an attempt to determine the MPC values for the MSSA ATCC control strain (ATCC 29213) against gatifloxacin.

Using gatifloxacin E-test strips, the concentration required to inhibit the growth of MSSA (ATCC 29213) at a bacterial concentration of $10^9$ CFUs was experimentally determined to be 0.25 μg/ml in two separate assays. The MPC values for MSSA (ATCC 29213) against gatifloxacin, determined using both the traditional and microbroth dilution MPC methods, were 8 μg/ml and ≥8 μg/ml respectively. Therefore, the E-test determined MPC values for MSSA (ATCC 29213) against gatifloxacin are not consistent with MPC values determined using either the traditional- or modified- MPC method.

In further experiments, we determined the inhibitory concentration values (μg/ml) of gatifloxacin for different bacterial concentrations (i.e. $10^1$ to $10^9$ CFU/ml) of MSSA (ATCC 29213) using gatifloxacin E-test strips. Bacterial suspensions of MSSA (ATCC 29213) with cellular concentrations of $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$, and $10^9$

| | *S. pneumoniae* (ATCC 49619) | | | |
|---|---|---|---|---|
| | Traditional Method[a] | | Microbroth Dilution Method | |
| **Drug** | **MIC (µg/ml)** | **MPC (µg/ml)** | **MIC (µg/ml)** | **MPC (µg/ml)** |
| **Gatifloxacin** | 0.25 | 0.5 | 0.25 | 0.5 |
| **Moxifloxacin** | 0.125 | 0.5 | 0.063 | 0.5 |
| | **MSSA (ATCC 29213)** | | | |
| **Gatifloxacin** | 0.125 | 8 | 0.125 | ≥8 |
| **Moxifloxacin** | 0.031 | 4 | 0.031 | >2 |

**Table 3.1.2.1.2: Comparative MIC and MPC values (µg/ml) as determined by the traditional method[a] and the microbroth dilution method for *S. pneumoniae* (ATCC 49619) and MSSA (ATCC 29213) against gatifloxacin and moxifloxacin.**

[a] Agar Dilution Method

MIC = Minimum Inhibitory Concentration; MPC = Mutant Prevention Concentration; MSSA = Methicillin-susceptible *Staphylococcus aureus*

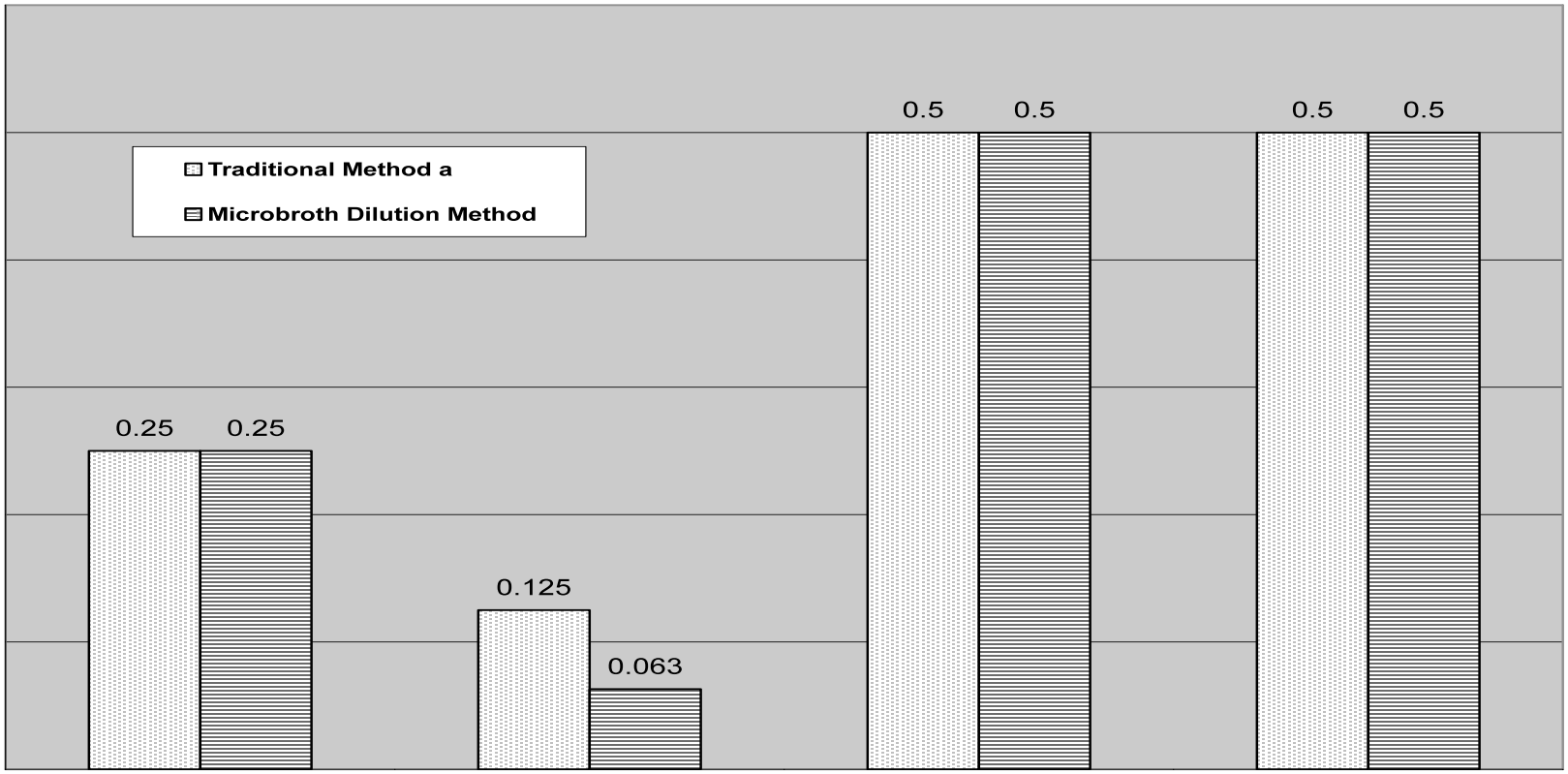


**Figure 3.1.2.1.1: Comparative MIC and MPC values (µg/ml) as determined by the traditional method[a] and the microbroth dilution method for *S. pneumoniae* (ATCC 49619) against gatifloxacin and moxifloxacin.**

[a] Agar Dilution Method

MIC = Minimum Inhibitory Concentration; MPC = Mutant Prevention Concentration

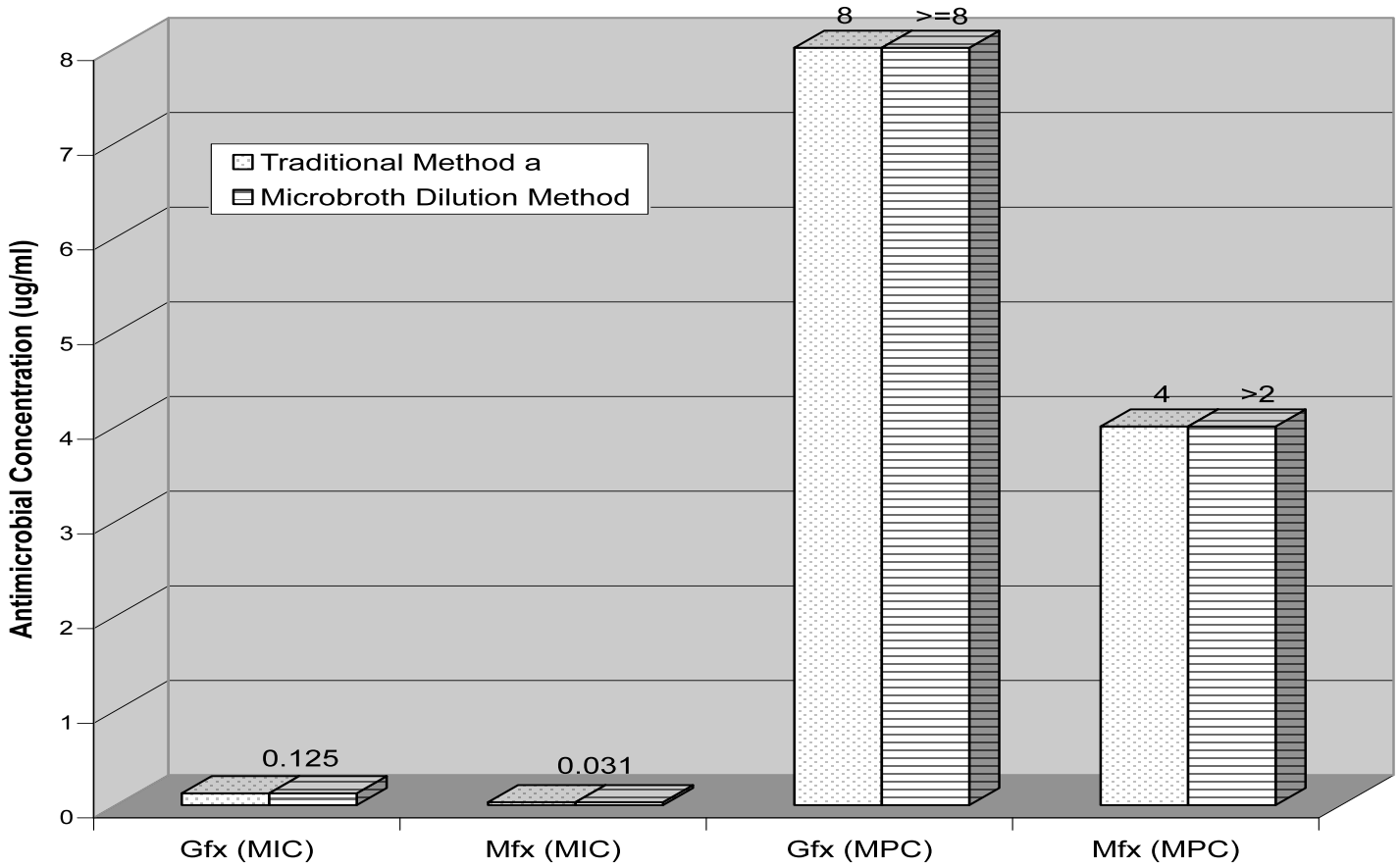


**Figure 3.1.2.1.2: Comparative MIC and MPC values (µg/ml) as determined by the traditional method[a] and the microbroth dilution method for MSSA (ATCC 29213) against gatifloxacin and moxifloxacin.**

[a] Agar Dilution Method

MIC = Minimum Inhibitory Concentration; MPC = Mutant Prevention Concentration; MSSA = Methicillin-susceptible *Staphylococcus aureus*

CFU/ml were applied to agar plates and a gatifloxacin E-test strip was subsequently added before incubation for 18 to 24 hours under appropriate conditions. The assay was performed in duplicate. To ensure that the appropriate concentration of bacteria was added to each agar plate, a colony count of viable cells was performed on each bacterial concentration. When 100 µl of organism is added to the agar plate, the actual bacterial concentration on the plate is 1:10 the initial concentration in the tube (and the concentration of the viable count). For example, if 100 µl of a bacterial suspension of $10^9$ CFUs is added to the plate, the actual bacterial suspension of that plate will be $10^8$ CFUs. Therefore in order to determine the MPC of a bacterial suspension of $10^9$ CFUs, an initial bacterial suspension of $10^{10}$ CFUs is required.

In the first assay, the resulting inhibitory concentrations (µg/ml) were 0.064, 0.064, 0.094, 0.094, 0.125, 0.19, and 0.25 for bacterial burdens of $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$, and $10^9$ CFUs respectively. Similar results were observed in the second assay with inhibitory concentrations (µg/ml) of 0.064, 0.064, 0.064, 0.125, 0.125, 0.19, and 0.25 for bacterial burdens of $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$, and $10^9$ CFUs respectively (Figure 3.1.3.1c - i). There were too few colonies to determine inhibitory concentrations with the E-test strips at bacterial burdens of $10^1$ and $10^2$ CFUs (Figure 3.1.3.1a and b). When these inhibitory concentrations were compared to the inhibitory concentrations determined with the microbroth dilution method, there was a noticeable discrepancy in values (Table 3.1.3.1 and Table 3.1.3.2). The inhibitory values were similar for both methods until higher bacterial burdens were used (i.e. $10^7$ to $10^9$ CFUs) indicating that the E-test method is not a good method for determining inhibitory concentrations of higher-density bacterial suspensions.

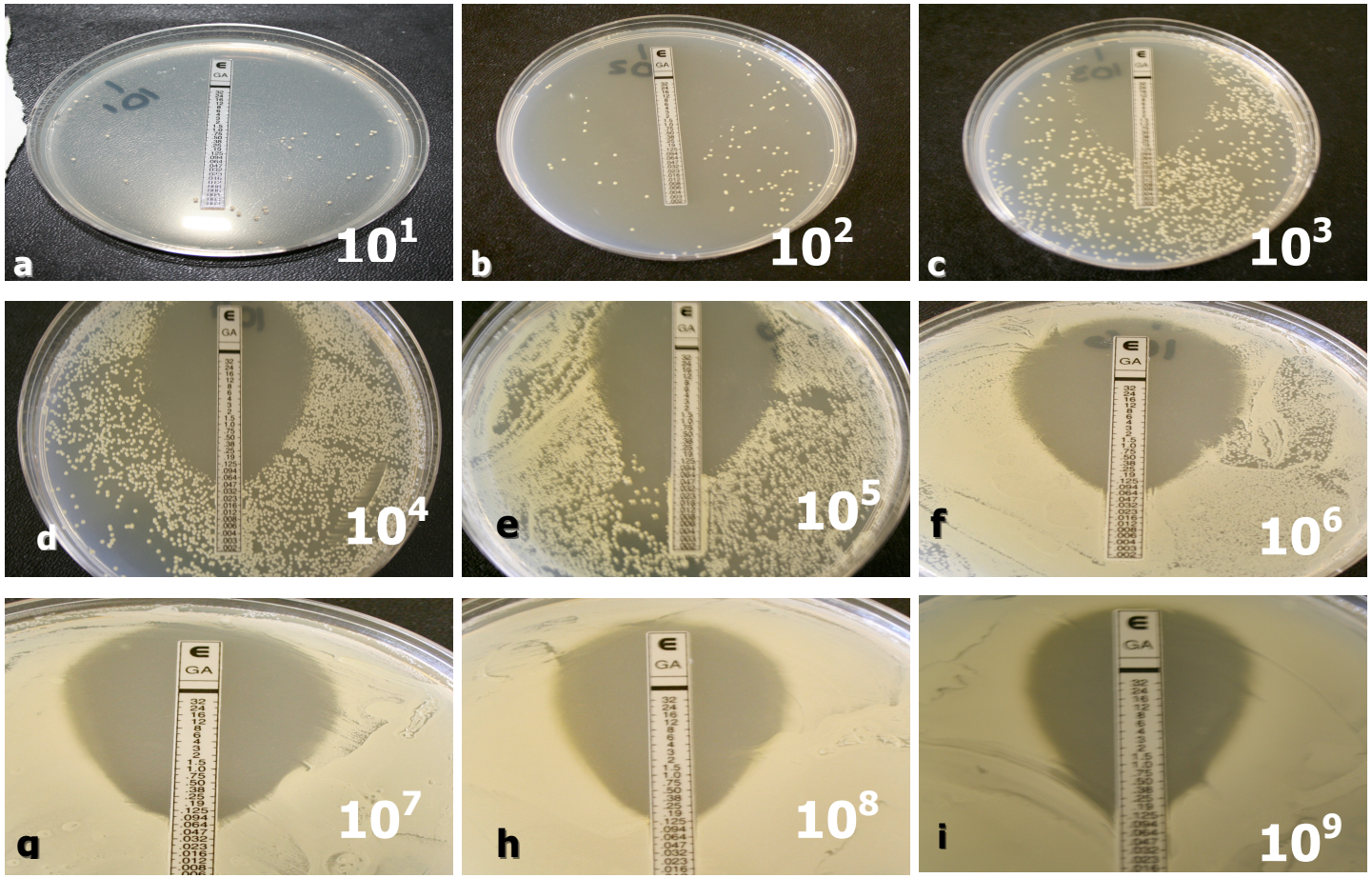


**Figure 3.1.3.1: Photos of E-test strips containing a concentration gradient of gatifloxacin on plates with different bacterial concentrations of MSSA (ATCC 29213).**
MSSA = Methicillin-susceptible *Staphylococcus aureus*

| Bacterial Concentration (CFUs) | Inhibitory Concentrations (µg/ml) | | Viable count of initial tube (CFU/ml) |
|---|---|---|---|
| | E-test Method | Microbroth Dilution Method | |
| $10^1$ | too few colonies | 0.063 | $4.23 \times 10^2$ |
| $10^2$ | too few colonies | 0.063 | $3.73 \times 10^3$ |
| $10^3$ | 0.064 | 0.063 | $2.90 \times 10^4$ |
| $10^4$ | 0.064 | 0.063 | $3.63 \times 10^5$ |
| $10^5$ | 0.094 | 0.125 | $5.10 \times 10^6$ |
| $10^6$ | 0.094 | 0.125 | $5.37 \times 10^7$ |
| $10^7$ | 0.125 | 4 | $4.93 \times 10^8$ |
| $10^8$ | 0.19 | ≥8 | $6.53 \times 10^9$ |
| $10^9$ | 0.25 | ≥8 | $6.97 \times 10^{10}$ |

**Table 3.1.3.1: Comparative inhibitory concentrations (µg/ml) for MSSA (ATCC 29213) against gatifloxacin determined using two methods: E-test method and microbroth dilution method. First assay.**



| Bacterial Concentration (CFUs) | Inhibitory Concentrations (µg/ml) | | Viable count of initial tube (CFU/ml) |
|---|---|---|---|
| | E-test Method | Microbroth Dilution Method | |
| $10^1$ | too few colonies | 0.063 | $2.73 \times 10^2$ |
| $10^2$ | too few colonies | 0.063 | $2.73 \times 10^3$ |
| $10^3$ | 0.064 | 0.063 | $2.70 \times 10^4$ |
| $10^4$ | 0.064 | 0.063 | $2.93 \times 10^5$ |
| $10^5$ | 0.064 | 0.125 | $3.47 \times 10^6$ |
| $10^6$ | 0.125 | 0.125 | $4.30 \times 10^7$ |
| $10^7$ | 0.125 | 4 | $5.00 \times 10^8$ |
| $10^8$ | 0.19 | ≥8 | $5.63 \times 10^9$ |
| $10^9$ | 0.25 | ≥8 | $6.00 \times 10^{10}$ |

**Table 3.1.3.2: Comparative inhibitory concentrations (µg/ml) for MSSA (ATCC 29213) against gatifloxacin determined using two methods: E-test method and microbroth dilution method. Second assay.**

### 3.1.4 Linear Regression

To determine whether it would be possible to extrapolate MPC values from the MIC values for specific bacterial pathogens against specific antimicrobial agents, we calculated the correlation coefficients ($r^2$) for five bacterial species against several flouroquinolones and for *S. pneumoniae* against several fluoroquinolones and three macrolides [82]. Linear regression was used to calculate the $r^2$ values. Data were from published and unpublished studies of clinical isolates. The $r^2$ values, shown in Table 3.1.4.1, are low (below 0.5) for fluoroquinolones with *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *S. aureus*, and *S. pneumoniae* (an exception was levofloxacin with *K. pneumoniae* [$r^2 = 0.7$]). The $r^2$ values for the three macrolides with *S. pneumoniae* were slightly above 0.5. The low $r^2$ values indicate that it is not possible to extrapolate a MPC value from a MIC value using simple linear regression.

## 3.2 The Impact of Media on MPC Testing

Whilst conducting MPC testing (traditional method) of tigecycline against clinical isolates of *S. pneumoniae* we measured MPC values that were unexpectedly higher than the corresponding MIC values for each isolate. The MPC results for most bacteria-antimicrobial agent combinations are generally 2 to 8 fold greater than the corresponding MIC results [83]. However, in our testing of tigecycline against *S. pneumoniae* we measured MPC values that were ≥512 fold higher than the corresponding MIC values. We designed several experiments to determine whether the media, specifically blood in the media, was having an impact on MPC results.

| Bacterial species | Compound tested | $r^2$ for MPC/MIC | No. of isolates |
|---|---|---|---|
| ***E. coli*** | Garenoxacin | 0.26 | 44 |
| | Gatifloxacin | 0.03 | 44 |
| | Gemifloxacin | 0.1 | 44 |
| | Levofloxacin | 0.02 | 43 |
| | Moxifloxacin | 0.03 | 40 |
| ***K. pneumoniae*** | Garenoxacin | 0.09 | 39 |
| | Gatifloxacin | 0.34 | 40 |
| | Gemifloxacin | 0.15 | 39 |
| | Levofloxacin | 0.7 | 41 |
| | Moxifloxacin | 0.18 | 40 |
| ***P. aeruginosa*** | Ciprofloxacin | 0.41 | 151 |
| | Levofloxacin | 0.43 | 151 |
| ***S. aureus*** | Ciprofloxacin | 0.11 | 21 |
| | Garenoxacin | 0.08 | 82 |
| | Gatifloxacin | 0.04 | 218 |
| | Gemifloxacin | 0.05 | 218 |
| | Levofloxacin | 0.18 | 220 |
| | Moxifloxacin | 0.08 | 219 |
| ***S. pneumoniae*** | Garenoxacin | 0.31 | 524 |
| | Gatifloxacin | 0.28 | 516 |
| | Gemifloxacin | 0.29 | 495 |
| | Levofloxacin | 0.44 | 528 |
| | Moxifloxacin | 0.17 | 523 |
| | Azithromycin | 0.58 | 499 |
| | Erythromycin | 0.67 | 293 |
| | Clarithromycin | 0.67 | 278 |

**Table 3.1.4.1: Relationship between MICs and MPCs.**
MIC = Minimum Inhibitory Concentration; MPC = Mutant Prevention Concentration; $r^2$ = correlational coefficient [82]

### 3.2.1 *Streptococcus pneumoniae*

Using traditional methods, we measured the MIC and MPC values for tigecycline against 47 clinical isolates of *S. pneumoniae*. Traditional methods for MIC and MPC testing were the microbroth dilution method and the agar dilution method using tryptic soy agar (TSA) with 5% sheep red blood cells (SRBCs) respectively. The $MIC_{50}$ and $MPC_{50}$ values were defined as the lowest drug concentration preventing growth of 50% of bacterial strains while the $MIC_{90}$ and $MPC_{90}$ were defined as the lowest drug concentration preventing growth of 90% of bacterial strains. The $MIC_{50}$, $MIC_{90}$ and MIC range values (μg/ml) were 0.016, 0.031, and ≤0.008 – 0.125 respectively. The $MPC_{50}$, $MPC_{90}$, and MPC range values (μg/ml) were 8, ≥16, and ≤1 - ≥16 respectively. To investigate whether the presence of blood in the test media was influencing MPC results, we developed a novel formulation of a medium on which *S. pneumoniae* could grow without blood. The new test medium was developed in our lab by solidifying Todd-Hewitt Broth with 1.5% agarose; these plates were called solidified Todd-Hewitt Broth (sTHB) plates [80, 81].

MPC testing of tigecycline against the same 47 clinical isolates of *S. pneumoniae* was completed using sTHB plates (without blood) and the resulting $MPC_{50}$, $MPC_{90}$, and MPC range values (μg/ml) were 0.063, 0.5, and 0.063 – 0.5 respectively. These values were considerably lower than those measured using TSA + 5% SRBCs. In fact, the $MPC_{90}$ values measured using sTHB (without blood) were only 2 fold higher than the $MIC_{90}$ values of the same isolates. Recall, the $MPC_{90}$ values measured using TSA + 5% SRBCs were ≥512 fold higher than the corresponding $MIC_{90}$ values. Additional experiments were performed to determine whether the significantly lower MPC values

observed when using sTHB over TSA + 5% SRBCs were due to the absence of blood in sTHB or because of the sTHB itself. MPC testing was done with tigecycline against the 47 clinical isolates of *S. pneumoniae* using sTHB + 5% SRBCs. The resulting $MPC_{50}$, $MPC_{90}$, and MPC range values (μg/ml) using sTHB + 5% SRBCs were >8, >8, and 4 - ≥8 respectively, indicating the presence of some type of interaction between blood and tigecycline in the testing of *S. pneumoniae*. The results of all MPC and MIC testing are summarized in Table 3.2.1.1 and Figure 3.2.1.1.

### 3.2.2 *Staphylococcus aureus*

To investigate whether the presence of blood in the test media would influence MPC results for other organisms against tigecycline, MPC testing was done with tigecycline against clinical isolates of MSSA (n = 50) and MRSA (n = 50). First we used traditional methods (microbroth dilution method for MIC and agar dilution method for MPC testing) to determine the MIC and MPC values of tigecycline against both MSSA and MRSA isolates. Generally, when MPC testing is done with *S. aureus* isolates (MSSA and MRSA) TSA plates are created containing doubling dilutions of drug with no blood. For these experiments we completed traditional MPC testing using TSA plates without blood and also TSA plates with 5% SRBCs. We measured $MIC_{50}$, $MIC_{90}$, and MIC range values (μg/ml) of 0.063, 0.125, and 0.031 – 0.125 respectively for MSSA and 0.125, 0.5, and 0.063 – 1 respectively for MRSA. For MSSA isolates we measured $MPC_{50}$, $MPC_{90}$, and MPC range values (μg/ml) of 1, 2, and 1 – 4 respectively on TSA (no blood) compared to 0.5, 1, and 0.25 – 2 respectively on TSA + 5% SRBC. For MRSA isolates the $MPC_{50}$, $MPC_{90}$, and MPC range values (μg/ml) on TSA (without blood) were 1, 4, and 0.5 – 8 respectively compared to 0.5, 4, and 0.5 – 4 respectively on

| Test Media | $MPC_{50}$[a] (µg/ml) | $MPC_{90}$[b] (µg/ml) | MPC Range (µg/ml) |
|---|---|---|---|
| **TSA + 5% SRBC** | 8 | ≥16 | ≤1 - ≥16 |
| **sTHB** | 0.063 | 0.5 | 0.063 - 0.5 |
| **sTHB + 5% SRBC** | >8 | >8 | 4 - ≥8 |
| | $MIC_{50}$[a] (µg/ml) | $MIC_{90}$[b] (µg/ml) | MIC Range (µg/ml) |
| **Microbroth Dilution** | 0.016 | 0.031 | ≤0.008 - 0.125 |

**Table 3.2.1.1: Summary of MIC and MPC Results for tigecycline against 47 clinical isolates of *S. pneumoniae* measured using different media.**

[a] The drug concentration inhibiting 50% of isolates tested

[b] The drug concentration inhibiting 90% of isolates tested

MPC = Mutant Prevention Concentration; MIC = Minimum Inhibitory Concentration; TSA = tryptic soy agar; SRBC = sheep red blood cells; sTHB = solidified Todd-Hewitt Broth

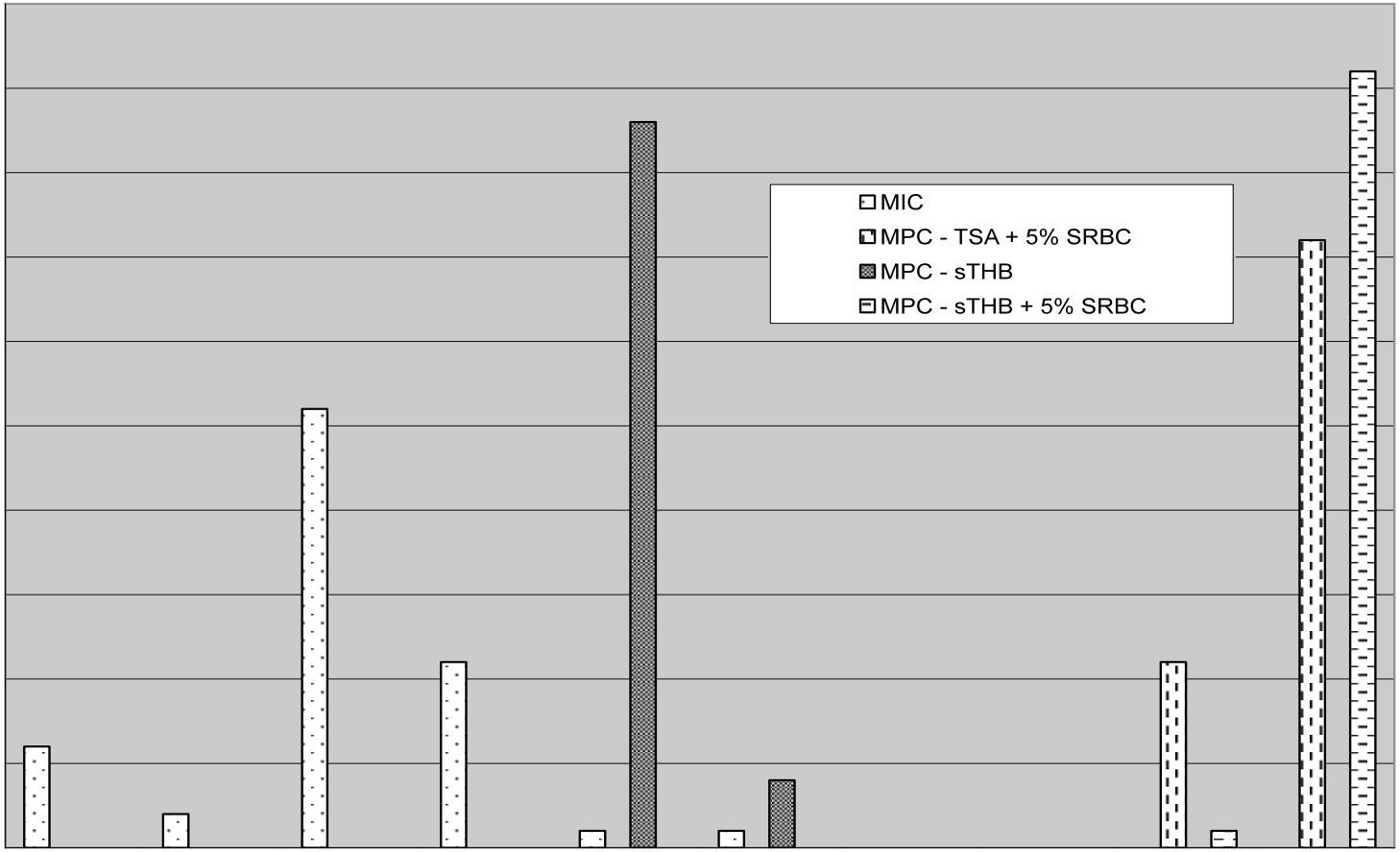


**Figure 3.2.1.1: Distribution values for 47 clinical isolates of *S. pneumoniae* against tigecycline.**
MPC = Mutant Prevention Concentration; MIC = Minimum Inhibitory Concentration; TSA = tryptic soy agar; SRBC = sheep red blood cells; sTHB = solidified Todd-Hewitt Broth

TSA + 5% SRBC (Table 3.2.2.1).

The distribution of all MIC and MPC values for tigecycline against 50 clinical isolates of MSSA (Figure 3.2.2.1) and for tigecycline against 50 clinical isolates of MRSA (Figure 3.2.2.2) demonstrate that the MPC values were the same or within one doubling dilution on TSA and TSA + 5% SRBC. The $MPC_{90}$ for tigecycline against MSSA on TSA was 16 fold higher than the corresponding $MIC_{90}$ while the $MPC_{90}$ on TSA + 5% SRBC was 8 fold higher than the corresponding $MIC_{90}$. The $MPC_{90}$ for tigecycline against MRSA was 4 fold higher than the corresponding $MIC_{90}$ for testing done using TSA and TSA + 5% SRBC. Therefore, tigecycline MPC values for MSSA and MRSA do not appear to be influenced by blood in the test media [84].

## 3.3 The Use of BAK in Conjunction with gatifloxacin and moxifloxacin

Benzalkonium chloride (BAK) is a cationic surface-acting agent presently being used as a preservative in the commercial preparation of Zymar® (0.3% gatifloxacin plus 0.005% BAK), an antimicrobial agent used to treat ocular infections. We assessed the impact of BAK on the MIC and MPC values of gatifloxacin and moxifloxacin against Gram-positive and Gram-negative organisms. We also determined the killing action of BAK alone and in conjunction with gatifloxacin against clinical isolates of MRSA.

### 3.3.1 MIC Results

Minimum inhibitory concentration (MIC) values were measured for clinical isolates of MRSA (n = 20), MSSA (n = 20), coagulase-negative staphylococci (CNS; n = 20), *S. pneumoniae* (n = 20), *P. aeruginosa* (n = 20), and *E. coli* (n = 20) against gatifloxacin and moxifloxacin with and without BAK and against BAK alone.

When conducting MIC assays with gatifloxacin or moxifloxacin and BAK the

| Organism | Test Media | $MPC_{50}$[a] (µg/ml) | $MPC_{90}$[b] (µg/ml) | MPC Range (µg/ml) |
|---|---|---|---|---|
| **MSSA** | TSA | 1 | 2 | 1 - 4 |
| | TSA + 5% SRBC | 0.5 | 1 | 0.25 - 2 |
| **MRSA** | TSA | 1 | 4 | 0.5 - 8 |
| | TSA + 5% SRBC | 0.5 | 4 | 0.5 - 4 |
| | | $MIC_{50}$[a] (µg/ml) | $MIC_{90}$[b] (µg/ml) | MIC Range (µg/ml) |
| **MSSA** | Microbroth Dilution | 0.063 | 0.125 | 0.031 - 0.125 |
| **MRSA** | Microbroth Dilution | 0.125 | 0.5 | 0.063 - 1 |



**Table 3.2.2.1: Summary of MIC and MPC results for tigecycline against 50 clinical isolates of MSSA and MRSA measured using different media.**

[a] The drug concentration inhibiting 50% of isolates tested

[b] The drug concentration inhibiting 90% of isolates tested

MIC = Minimum Inhibitory Concentration; MPC = Mutant Prevention Concentration; MSSA = Methicillin-susceptible *Staphylococcus aureus*; MRSA = Methicillin-resistant *Staphylococcus aureus*; TSA = tryptic soy agar; SRBC = sheep red blood cells

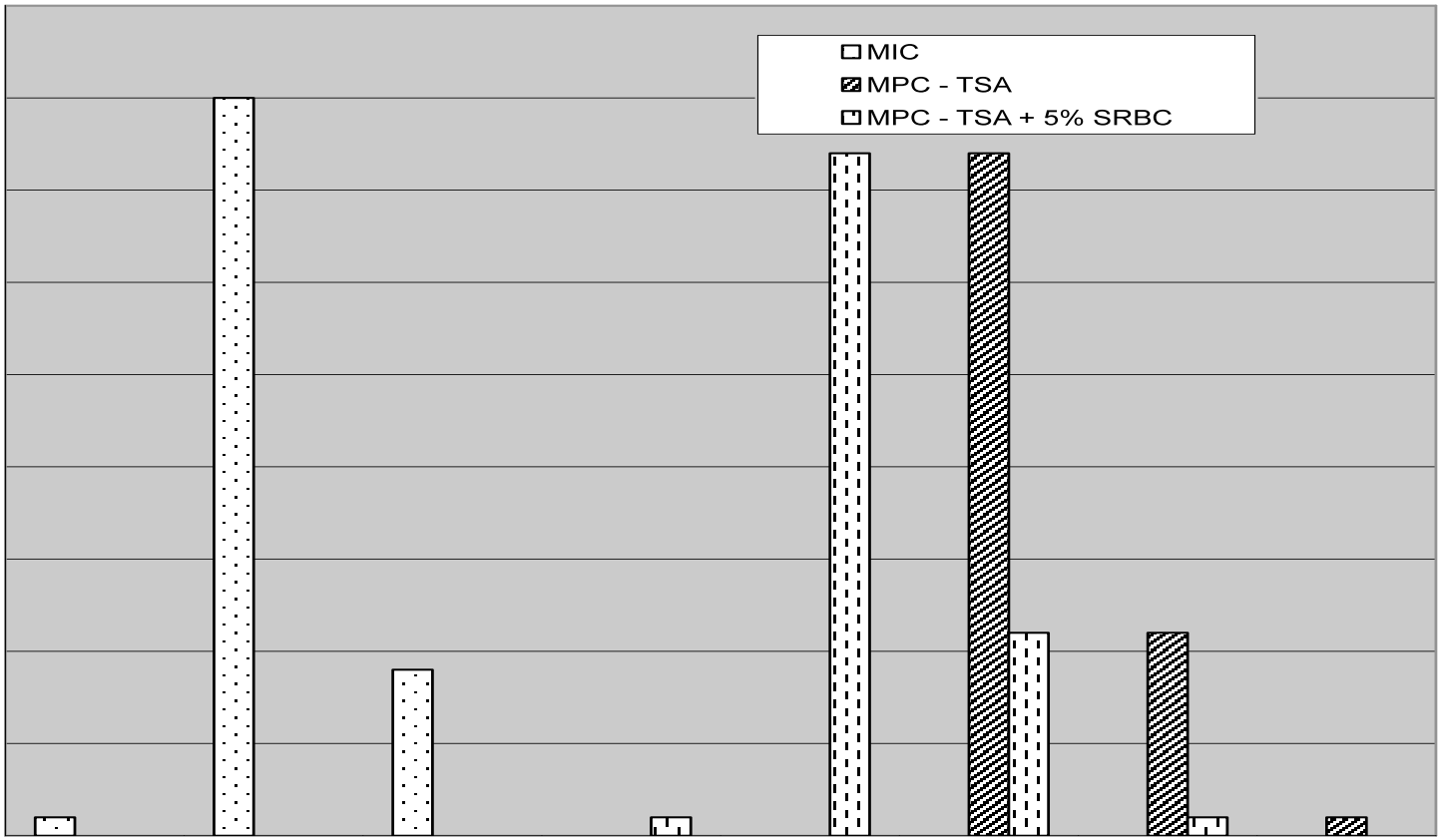


**Figure 3.2.2.1: Distribution values for 50 clinical isolates of MSSA against tigecycline.**
MPC = Mutant Prevention Concentration; MIC = Minimum Inhibitory Concentration; TSA = tryptic soy agar; SRBC = sheep red blood cells; MSSA = Methicillin-susceptible *Staphylococcus aureus*

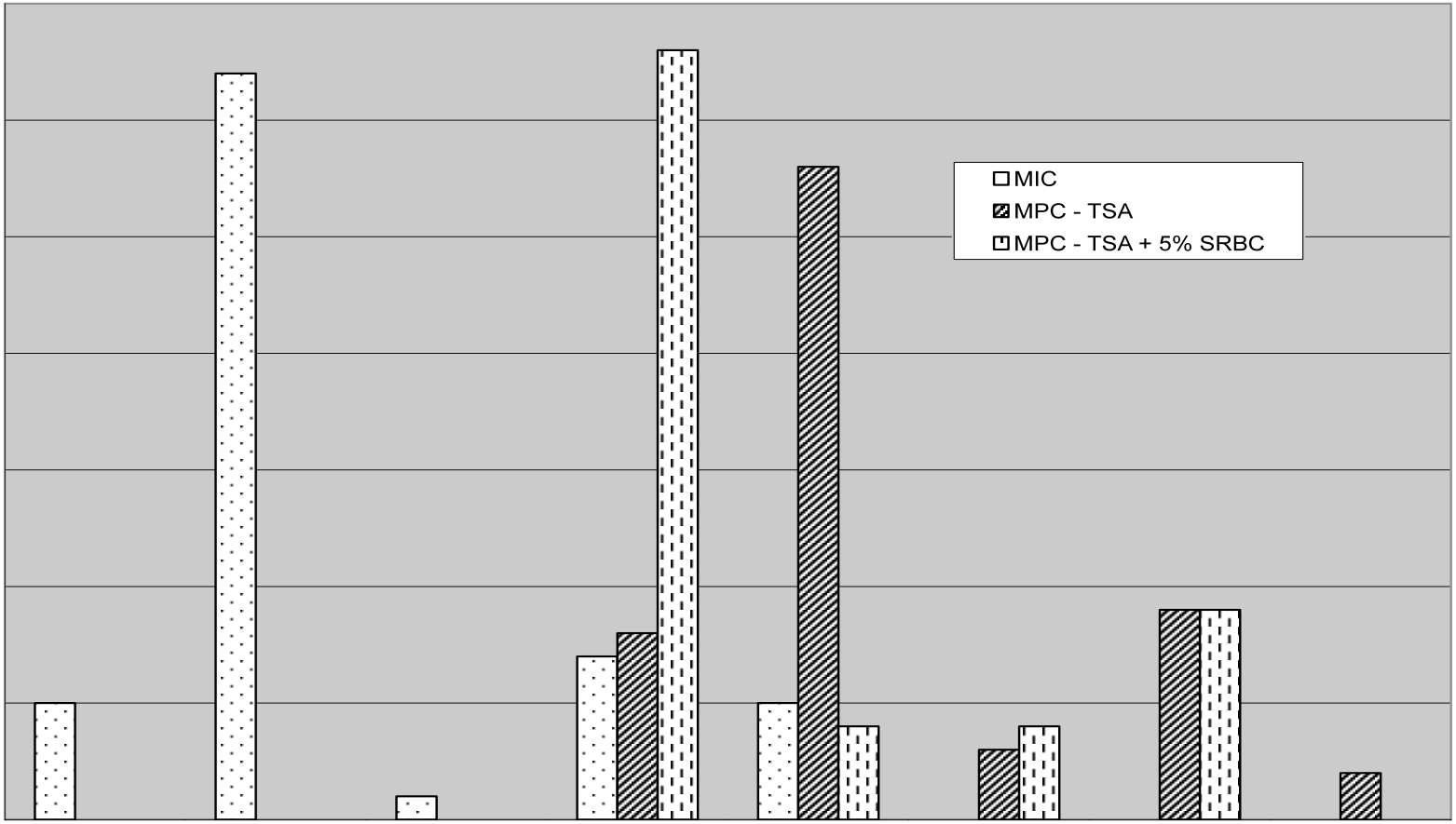


**Figure 3.2.2.2: Distribution values for 50 clinical isolates of MRSA against tigecycline.**
MPC = Mutant Prevention Concentration; MIC = Minimum Inhibitory Concentration; TSA = tryptic soy agar; SRBC = sheep red blood cells; MRSA = Methicillin-resistant *Staphylococcus aureus*

concentration of BAK was kept constant at 50 µg/ml; gatifloxacin and moxifloxacin were first serially diluted in 96-well microtitre panels and BAK was then added so the final concentration of BAK in each well was 50 µg/ml. A BAK concentration of 50 µg/ml was selected because it is the concentration of BAK present in the commercial preparation of Zymar® (i.e. 0.005% BAK). The lowest drug concentration preventing growth of 90% of bacterial strains was recorded as the $MIC_{90}$. The $MIC_{90}$ values of gatifloxacin and moxifloxacin alone and with BAK and of BAK alone against the study organisms are presented in Table 3.3.1.1.

The $MIC_{90}$ values (µg/ml) for BAK against Gram-positive organisms ranged from 1.563 to 3.125 (MRSA = 3.125 µg/ml; MSSA = 1.563 µg/ml; CNS = 3.125 µg/ml; *S. pneumoniae* = 1.563 µg/ml). BAK was less active against Gram-negative organisms with $MIC_{90}$ values (µg/ml) of 12.5 for *E. coli* and 50 for *P. aeruginosa*. The $MIC_{90}$ values (µg/ml) for gatifloxacin and moxifloxacin against MRSA were both >4; against MSSA they were 2 and 1 respectively; 2 and 4 respectively against CNS; 1 and 0.125 respectively against *S. pneumoniae*; 2 and 4 respectively for *P. aeruginosa*; and they were both >2 for *E. coli*. All study organisms, with the exception of *S. pneumoniae*, were equally susceptible to either gatifloxacin or moxifloxacin with $MIC_{90}$ values falling within one doubling-dilution when either antibiotic was used. *S. pneumoniae* was more susceptible to moxifloxacin ($MIC_{90}$ = 0.125 µg/ml) than gatifloxacin ($MIC_{90}$ = 1 µg/ml).

In the presence of BAK (50 µg/ml), the $MIC_{90}$ values of gatifloxacin and moxifloxacin against all four Gram-positive organisms tested (MRSA, MSSA, CNS, and *S. pneumoniae*) were reduced to ≤0.008 µg/ml; a 100-fold to >500-fold reduction in $MIC_{90}$ values compared to those measured with gatifloxacin or moxifloxacin alone. The

| Organism | N | $MIC_{90}$[a] (µg/ml) | | | | |
|---|---|---|---|---|---|---|
| | | BAK | Gfx | Gfx + BAK[b] | Mfx | Mfx + BAK[b] |
| **MRSA** | 20 | 3.125 | >4 | ≤0.008 | >4 | ≤0.008 |
| **MSSA** | 20 | 1.563 | 2 | ≤0.008 | 1 | ≤0.008 |
| **CNS** | 20 | 3.125 | 2 | ≤0.008 | 4 | ≤0.008 |
| ***S. pneumoniae*** | 20 | 1.563 | 1 | ≤0.008 | 0.125 | ≤0.008 |
| ***P. aeruginosa*** | 20 | 50 | 2 | 0.5 | 4 | 1 |
| ***E. coli*** | 20 | 12.5 | >2 | ≤0.002 | >2 | ≤0.002 |

**Table 3.3.1.1: Summary of $MIC_{90}$ results for 20 clinical isolates of Gram-positive and Gram-negative organisms against gatifloxacin and moxifloxacin with and without BAK and against BAK alone.**

[a] The drug concentration inhibiting 90% of isolates tested

[b] BAK was added at a concentration of 50 µg/ml

MIC = Minimum Inhibitory Concentration; MRSA = Methicillin-resistant *Staphylococcus aureus*; MSSA = Methicillin-susceptible *Staphylococcus aureus*; CNS = coagulase-negative staphylococci; BAK = Benzalkonium chloride; Gfx = Gatifloxacin; Mfx = Moxifloxacin

$MIC_{90}$ values of gatifloxacin and moxifloxacin against Gram-negative organisms were also substantially lower in the presence of BAK (50 μg/ml); $MIC_{90}$ values (μg/ml) of 0.5 and ≤0.002 were measured for *P. aeruginosa* and *E. coli* respectively against gatifloxacin with BAK (50 μg/ml) and $MIC_{90}$ values (μg/ml) of 1 and ≤0.002 for *P. aeruginosa* and *E. coli* respectively against moxifloxacin with BAK (50 μg/ml). The $MIC_{90}$ of gatifloxacin and moxifloxacin against *E. coli* decreased by >500-fold in the presence of 50 μg/ml of BAK while the $MIC_{90}$ of gatifloxacin and moxifloxacin against *P. aeruginosa* decreased by 4-fold in the presence of 50 μg/ml of BAK.

There was a substantial decrease in the $MIC_{90}$ values of gatifloxacin and moxifloxacin against Gram-positive and Gram-negative organisms in the presence of 50 μg/ml of BAK, however, to determine the actual concentration of BAK required to achieve these substantially reduced $MIC_{90}$ values, MIC testing was completed with gatifloxacin and moxifloxacin against MRSA, MSSA, CNS, *S. pneumoniae*, *P. aeruginosa*, and *E. coli* in the presence of differing concentrations of BAK (0.391 μg/ml to 50 μg/ml). The $MIC_{90}$ values of gatifloxacin and moxifloxacin alone and with BAK at concentrations of 0.391 μg/ml to 50 μg/ml against the study organisms are presented in Tables 3.3.1.2 and 3.3.1.3 respectively. The $MIC_{90}$ of gatifloxacin and moxifloxacin against MRSA, MSSA, and *S. pneumoniae* decreased to ≤0.008 μg/ml in the presence of 3.125 μg/ml of BAK and the $MIC_{90}$ of gatifloxacin and moxifloxacin against CNS decreased to ≤0.008 μg/ml in the presence of 6.25 μg/ml of BAK [49]. Higher concentrations of BAK were needed to substantially reduce the $MIC_{90}$ values of gatifloxacin and moxifloxacin against the Gram-negative organisms *P. aeruginosa* and *E. coli*; in the presence of 50 μg/ml of BAK the $MIC_{90}$ of gatifloxacin and moxifloxacin

| Organism | $MIC_{90}$[a] (µg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | BAK | Gfx | Gfx + 50µg/ml BAK | Gfx + 25µg/ml BAK | Gfx + 12.5µg/ml BAK | Gfx + 6.25µg/ml BAK | Gfx + 3.125µg/ml BAK | Gfx + 1.563µg/ml BAK | Gfx + 0.781µg/ml BAK | Gfx + 0.391µg/ml BAK |
| **MRSA** | 3.125 | >4 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | 8 | >8 | >8 |
| **MSSA** | 1.563 | 2 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | 0.016 | 0.5 | 1 |
| **CNS** | 3.125 | 2 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | 0.5 | 2 | 4 | 4 |
| ***S. pneumoniae*** | 1.563 | 1 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | 0.063 | 0.5 | 0.5 |
| ***P. aeruginosa*** | 50 | 2 | 0.5 | 4 | ND | ND | ND | ND | ND | ND |
| ***E. coli*** | 12.5 | >2 | ≤0.002 | ≤0.002 | ≤0.002 | 2 | >2 | >2 | ND | ND |



**Table 3.3.1.2: Summary of $MIC_{90}$ results for 20 clinical isolates of Gram -positive and Gram-negative organisms against gatifloxacin, BAK, and gatifloxacin with different concentrations of BAK.**

[a] The drug concentration inhibiting 90% of isolates tested

MIC = Minimum Inhibitory Concentration; MRSA = Methicillin-resistant *Staphylococcus aureus*; MSSA = Methicillin-susceptible *Staphylococcus aureus*; CNS = coagulase-negative staphylococci; BAK = Benzalkonium chloride; Gfx = Gatifloxacin; ND = no data

| Organism | $MIC_{90}$[a] (µg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **BAK** | **Mfx** | **Mfx + 50µg/ml BAK** | **Mfx + 25µg/ml BAK** | **Mfx + 12.5µg/ml BAK** | **Mfx + 6.25µg/ml BAK** | **Mfx + 3.125µg/ml BAK** | **Mfx + 1.563µg/ml BAK** | **Mfx + 0.781µg/ml BAK** | **Mfx + 0.391µg/ml BAK** |
| **MRSA** | 3.125 | >4 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | 4 | 4 | 4 |
| **MSSA** | 1.563 | 1 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | 0.25 | 0.5 |
| **CNS** | 3.125 | 4 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | 0.25 | 2 | 2 | 2 |
| ***S. pneumoniae*** | 1.563 | 0.125 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | ≤0.008 | 0.031 | 0.125 | 0.125 |
| ***P. aeruginosa*** | 50 | 4 | 1 | 4 | ND | ND | ND | ND | ND | ND |
| ***E. coli*** | 12.5 | >2 | ≤0.002 | ≤0.002 | ≤0.002 | 2 | >2 | >2 | ND | ND |

**Table 3.3.1.3: Summary of $MIC_{90}$ results for 20 clinical isolates of Gram-positive and Gram-negative organisms against moxifloxacin, BAK, and moxifloxacin with different concentrations of BAK.**

[a] The drug concentration inhibiting 90% of isolates tested

MIC = Minimum Inhibitory Concentration; MRSA = Methicillin-resistant *Staphylococcus aureus*; MSSA = Methicillin-susceptible *Staphylococcus aureus*; CNS = coagulase-negative staphylococci; BAK = Benzalkonium chloride; Mfx = Moxifloxacin; ND = no data

against *P. aeruginosa* was reduced to 0.5 μg/ml and 1 μg/ml respectively while 12.5 μg/ml of BAK was required to reduce the $MIC_{90}$ of gatifloxacin and moxifloxacin against *E. coli* to ≤0.002 μg/ml.

### 3.3.2 MPC Results

Mutant prevention concentration (MPC) values were measured for gatifloxacin and moxifloxacin, in the presence and absence of BAK, against clinical isolates of fluoroquinolone-resistant MRSA (n = 9) and one commercially available MSSA strain, ATCC 29213. The traditional agar dilution method was used to determine MPC values (Section 2.3.2). The $MPC_{90}$ of gatifloxacin against MRSA isolates was similar to that of moxifloxacin (≥4 μg/ml) and the $MPC_{90}$ of BAK against the same MRSA isolates was 10 μg/ml (Table 3.3.2.1). The $MPC_{90}$ values remained ≥4 μg/ml for gatifloxacin and moxifloxacin in the presence of 5 μg/ml BAK, however, in the presence of 7 μg/ml BAK, the $MPC_{90}$ for moxifloxacin against MRSA decreased to ≤0.004 μg/ml. In the presence of 9 μg/ml BAK, the $MPC_{90}$ for gatifloxacin against MRSA decreased to ≤0.004 μg/ml. The addition of BAK at concentrations from 7 μg/ml to 9μg/ml lowered the $MPC_{90}$ (μg/ml) of gatifloxacin (≤0.004) and moxifloxacin (≤0.004) against MRSA by 1000-fold compared to gatifloxacin (≥4) or moxifloxacin (≥4) alone. The presence of BAK at concentrations from 5 μg/ml to 7 μg/ml also decreased the $MPC_{90}$ (μg/ml) of gatifloxacin (≤0.004) and moxifloxacin (≤0.004) against the commercially available MSSA strain, ATCC 29213, by 1000-fold compared to gatifloxacin (≥4) or moxifloxacin (≥4) alone (Table 3.3.2.1).

| Organism | $MIC_{90}$[a] (µg/ml) | | $MPC_{90}$[a] (µg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | BAK | Gfx | BAK | Gfx | Gfx + 5µg/ml BAK | Gfx + 7µg/ml BAK | Gfx + 8µg/ml BAK | Gfx +9µg/ml BAK | Gfx + 10µg/ml BAK |
| MRSA (n = 9) | 3.125 | ≥4 | 10 | ≥4 | ≥4 | ≥4 | ≥4 | ≤0.004 | ≤0.004 |
| MSSA (ATCC 29213) | 0.781 | 0.063 | 7 | ≥4 | ≥4 | ≤0.004 | ≤0.004 | ≤0.004 | ≤0.004 |
| | $MIC_{90}$[a] (µg/ml) | | $MPC_{90}$[a] (µg/ml) | | | | | | |
| Organism | BAK | Mfx | BAK | Mfx | Mfx + 5µg/ml BAK | Mfx + 7µg/ml BAK | Mfx + 8µg/ml BAK | Mfx +9µg/ml BAK | Mfx + 10µg/ml BAK |
| MRSA (n = 9) | 3.125 | 4 | 10 | ≥4 | ≥4 | ≤0.004 | ≤0.004 | ≤0.004 | ≤0.004 |
| MSSA (ATCC 29213) | 0.781 | 0.031 | 7 | ≥4 | ≤0.004 | ≤0.004 | ≤0.004 | ≤0.004 | ≤0.004 |

**Table 3.3.2.1:Summary of $MIC_{90}$ and $MPC_{90}$ values for nine clinical isolates of MRSA and one commercially available MSSA strain (ATCC 29213) against gatifloxacin, moxifloxacin, BAK, and gatifloxacin and moxifloxacin with different concentrations of BAK.**

[a] The drug concentration inhibiting 90% of isolates tested

MIC = Minimum Inhibitory Concentration; MPC = Mutant Prevention Concentration; MRSA = Methicillin-resistant *Staphylococcus aureus*; MSSA = Methicillin-susceptible *Staphylococcus aureus*; BAK = Benzalkonium chloride; Gfx = gatifloxacin; Mfx = moxifloxacin

### 3.3.3 Time-Kill Results

To determine the killing action of BAK, conventional time-kill studies were completed with differing concentrations of BAK against clinical isolates of MRSA (n = 4). The results of the time-kill studies are summarized in Tables 3.3.3.1 and 3.3.3.2 and Figures 3.3.3.1 and 3.3.3.2. The killing action of BAK against MRSA isolates was measured using five different BAK concentrations: 10 µg/ml, 15 µg/ml, 20 µg/ml, 25 µg/ml, and 50 µg/ml. The highest concentration of 50 µg/ml was chosen as it corresponds to the concentration of BAK present in the commercial preparation of Zymar® (0.005% BAK). The MRSA isolates were grown to a bacterial density of $10^5$ CFU/ml and colony counts were taken at 0, 5, 10, 15, 20, 25, 30, 60, 120, and 180 minutes after the addition of BAK. The $\log_{10}$-reduction and percent kill of viable cells was calculated at each time point for each bacterial isolate and averaged.

The $\log_{10}$-reduction (percent kill) of viable cells increased substantially with increasing BAK concentration. The $\log_{10}$-reduction (percent kill) of viable cells 180 minutes after the addition of BAK were -0.4 (-48.17%), -1.0 (-78.27%), -4.3 (-99.90%), -5.2 (-99.99%), and -5.5 (-100%) for BAK concentrations of 10 µg/ml, 15 µg/ml, 20 µg/ml, 25 µg/ml, and 50 µg/ml respectively. Bactericidal activity is associated with a greater than 3 $\log_{10}$-reduction of viable cells; in this case bactericidal activity was observed 10 minutes after the addition of 50 µg/ml BAK ($\log_{10}$-reduction = -5.5) to MRSA present at a concentration of $10^5$ CFU/ml. $\text{Log}_{10}$-reductions of -3.0 or greater were also calculated 60 minutes following the addition of 25 µg/ml BAK ($\log_{10}$-reduction = -3.7) and 120 minutes following the addition of 20 µg/ml BAK ($\log_{10}$-reduction = -3.7).

| Time (min) | $Log_{10}$ Reduction of Viable Cells | | | | |
|---|---|---|---|---|---|
| | BAK 10 µg/ml | BAK 15 µg/ml | BAK 20 µg/ml | BAK 25 µg/ml | BAK 50 µg/ml |
| **5** | 0.006 | 0.022 | -0.092 | -0.222 | -2.779 |
| **10** | 0.051 | -0.047 | -0.187 | -0.387 | -5.512 |
| **15** | -0.018 | -0.063 | -0.492 | -0.685 | -5.512 |
| **20** | -0.041 | -0.099 | -0.840 | -1.148 | -5.512 |
| **25** | -0.075 | -0.198 | -1.172 | -1.563 | -5.512 |
| **30** | -0.117 | -0.235 | -1.477 | -2.082 | -5.512 |
| **60** | -0.200 | -0.485 | -2.723 | -3.720 | -5.512 |
| **120** | -0.273 | -0.825 | -3.699 | -5.354 | -5.512 |
| **180** | -0.378 | -1.029 | -4.317 | -5.195 | -5.512 |



**Table 3.3.3.1: $Log_{10}$-reduction of viable cells using different concentrations of BAK against 4 clinical isolates of MRSA.**
BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

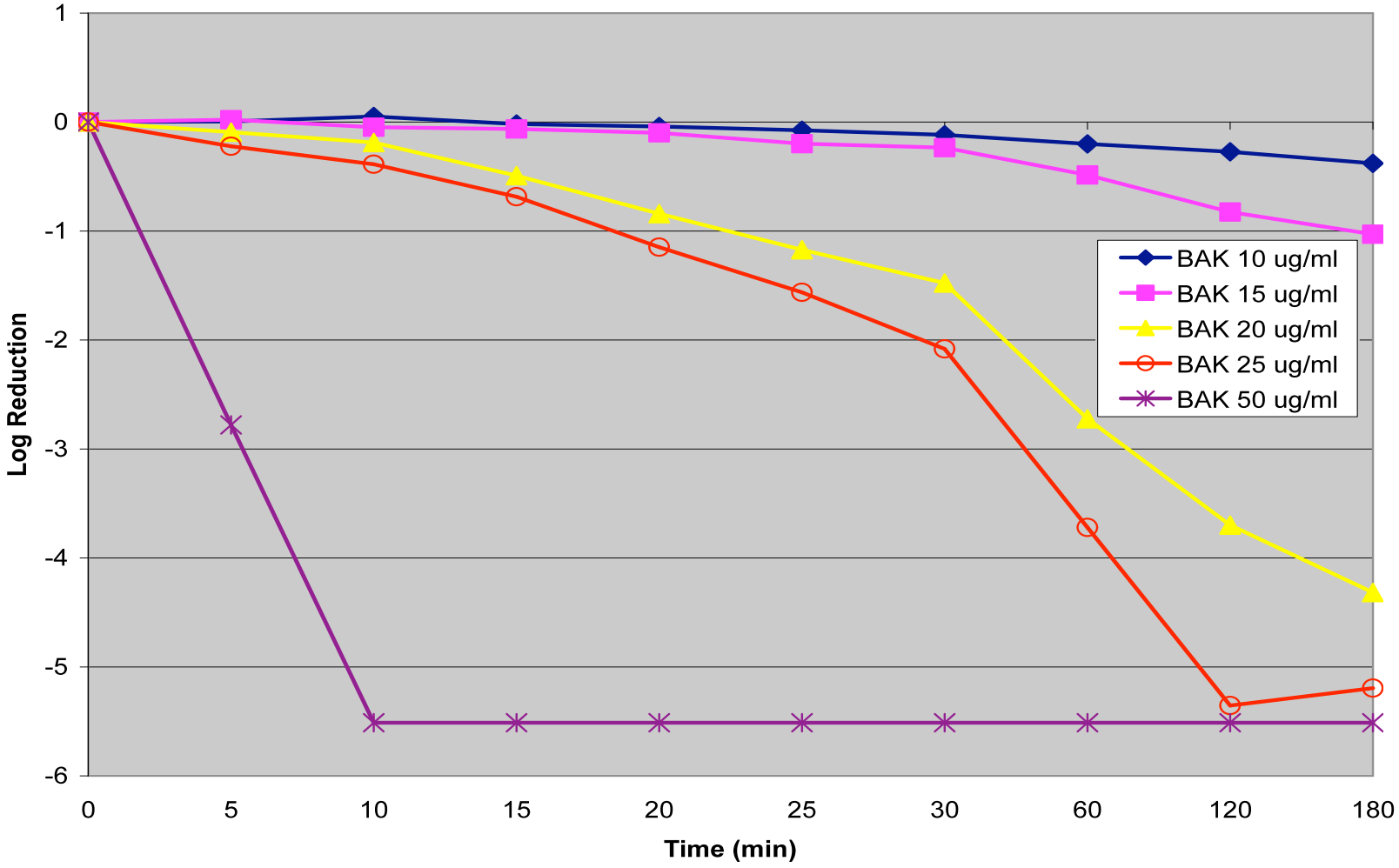


**Figure 3.3.3.1: $Log_{10}$-reduction of 4 clinical isolates of MRSA against differing concentrations of BAK.**
BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

| Time (min) | Percent Kill (%) | | | | |
|---|---|---|---|---|---|
| | BAK 10 µg/ml | BAK 15 µg/ml | BAK 20 µg/ml | BAK 25 µg/ml | BAK 50 µg/ml |
| **5** | 1.328 | 8.263 | -17.965 | -25.677 | -99.804 |
| **10** | 13.656 | -4.931 | -33.863 | -30.819 | -100.000 |
| **15** | -4.367 | -6.588 | -66.268 | -59.440 | -100.000 |
| **20** | -15.529 | -14.451 | -83.698 | -80.269 | -100.000 |
| **25** | -14.281 | -28.070 | -91.964 | -85.294 | -100.000 |
| **30** | -20.436 | -34.666 | -95.288 | -95.031 | -100.000 |
| **60** | -35.046 | -60.581 | -98.659 | -99.704 | -100.000 |
| **120** | -40.604 | -77.144 | -99.833 | -100.000 | -100.000 |
| **180** | -48.173 | -78.271 | -99.895 | -99.999 | -100.000 |



**Table 3.3.3.2: Percent Kill (%) of viable cells using different concentrations of BAK against 4 clinical isolates of MRSA.**
BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

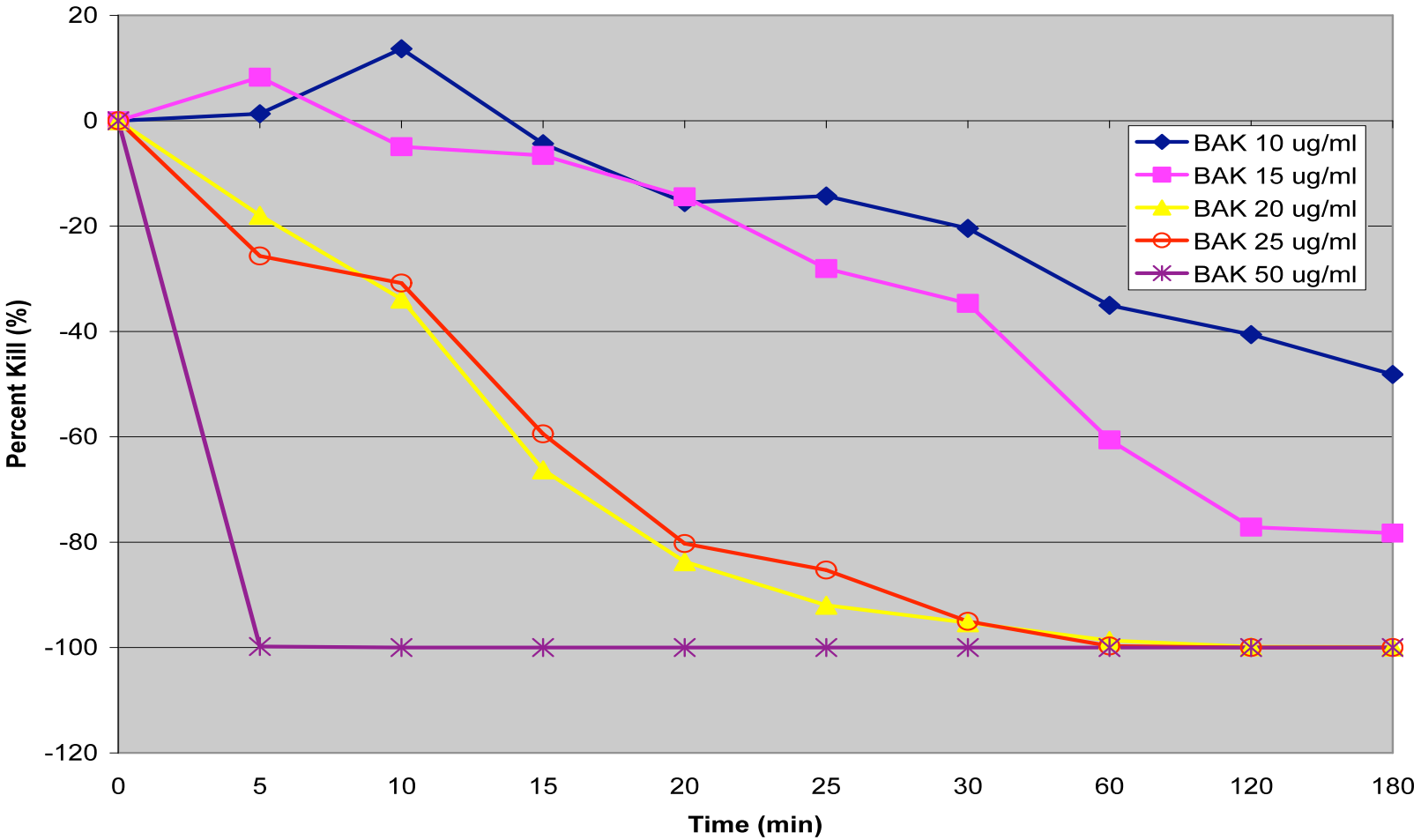


**Figure 3.3.3.2: Percent Kill (%) of 4 clinical isolates of MRSA against differing concentrations of BAK.**
BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

MIC and MPC studies (Sections 3.3.1 and 3.3.2) have demonstrated that the presence of BAK increases the antibacterial activity of gatifloxacin against MRSA isolates; lower MIC and MPC values were measured when gatifloxacin was used in conjunction with BAK than when used alone. In order to determine the impact of BAK on the killing action of gatifloxacin, conventional time-kill studies were conducted using gatifloxacin, BAK, and gatifloxacin plus BAK against clinical MRSA isolates (n = 2). The MRSA isolates were grown to a bacterial density of $10^5$ CFU/ml and colony counts were taken at 0, 5, 10, 15, 20, 25, 30, 60, 120, and 180 minutes after the addition of antimicrobial agent(s). The $\log_{10}$-reduction and percent kill of viable cells was calculated at each time point. Two clinical MRSA isolates were used for this time-kill assay and the results averaged. The results of the time-kill studies are summarized in Tables 3.3.3.3 and 3.3.3.4 and Figures 3.3.3.3 and 3.3.3.4.

The $\log_{10}$-reduction (percent kill) of viable cells 180 minutes after the addition of antimicrobial agent(s) to MRSA isolates (n = 2) was -1.6 (-76.08%), -5.0 (-99.99%), and -5.4 (100%) for gatifloxacin, BAK, and gatifloxacin plus BAK respectively. The concentration of gatifloxacin used in this assay was equivalent to the experimentally determined MIC value for each isolate and the concentration of BAK used was 25 μg/ml. Bactericidal activity is observed when BAK or gatifloxacin plus BAK are used against the clinical MRSA isolates with a greater than 5 $\log_{10}$-reduction in viable cells. A less than 2 $\log_{10}$-reduction in viable cells is observed when gatifloxacin is used alone against clinical MRSA isolates indicating reduced bactericidal activity.

Further conventional time-kill studies were completed to compare the killing

| Time (min) | $Log_{10}$-Reduction in Viable Cells | | |
|---|---|---|---|
| | Gfx (MIC µg/ml) | BAK (25 µg/ml) | Gfx (MIC µg/ml) + BAK (25µg/ml) |
| **5** | 0.026 | -0.084 | -0.072 |
| **10** | -0.042 | -0.130 | -0.169 |
| **15** | -0.111 | -0.392 | -0.290 |
| **20** | -0.114 | -0.740 | -0.589 |
| **25** | -0.187 | -1.051 | -0.986 |
| **30** | -0.286 | -1.563 | -1.716 |
| **60** | -0.602 | -3.304 | -4.825 |
| **120** | -1.121 | -5.199 | -5.387 |
| **180** | -1.621 | -4.960 | -5.387 |

**Table 3.3.3.3: $Log_{10}$-reduction of viable cells using gatifloxacin, BAK, and gatifloxacin plus BAK against 2 clinical isolates of MRSA.**
Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*



| Time (min) | Percent Kill (%) | | |
|---|---|---|---|
| | Gfx (MIC µg/ml) | BAK (25 µg/ml) | Gfx (MIC µg/ml) + BAK (25 µg/ml) |
| **5** | 7.614 | -16.299 | -14.793 |
| **10** | -8.426 | -21.029 | -29.284 |
| **15** | -20.789 | -54.519 | -45.898 |
| **20** | -12.377 | -78.108 | -71.825 |
| **25** | -19.826 | -84.648 | -88.667 |
| **30** | -29.158 | -94.853 | -97.597 |
| **60** | -41.098 | -99.860 | -99.998 |
| **120** | -53.764 | -99.999 | -100.000 |
| **180** | -76.079 | -99.999 | -100.000 |

**Table 3.3.3.4: Percent Kill (%) of viable cells using gatifloxacin, BAK, and gatifloxacin plus BAK against 2 clinical isolates of MRSA.**
Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

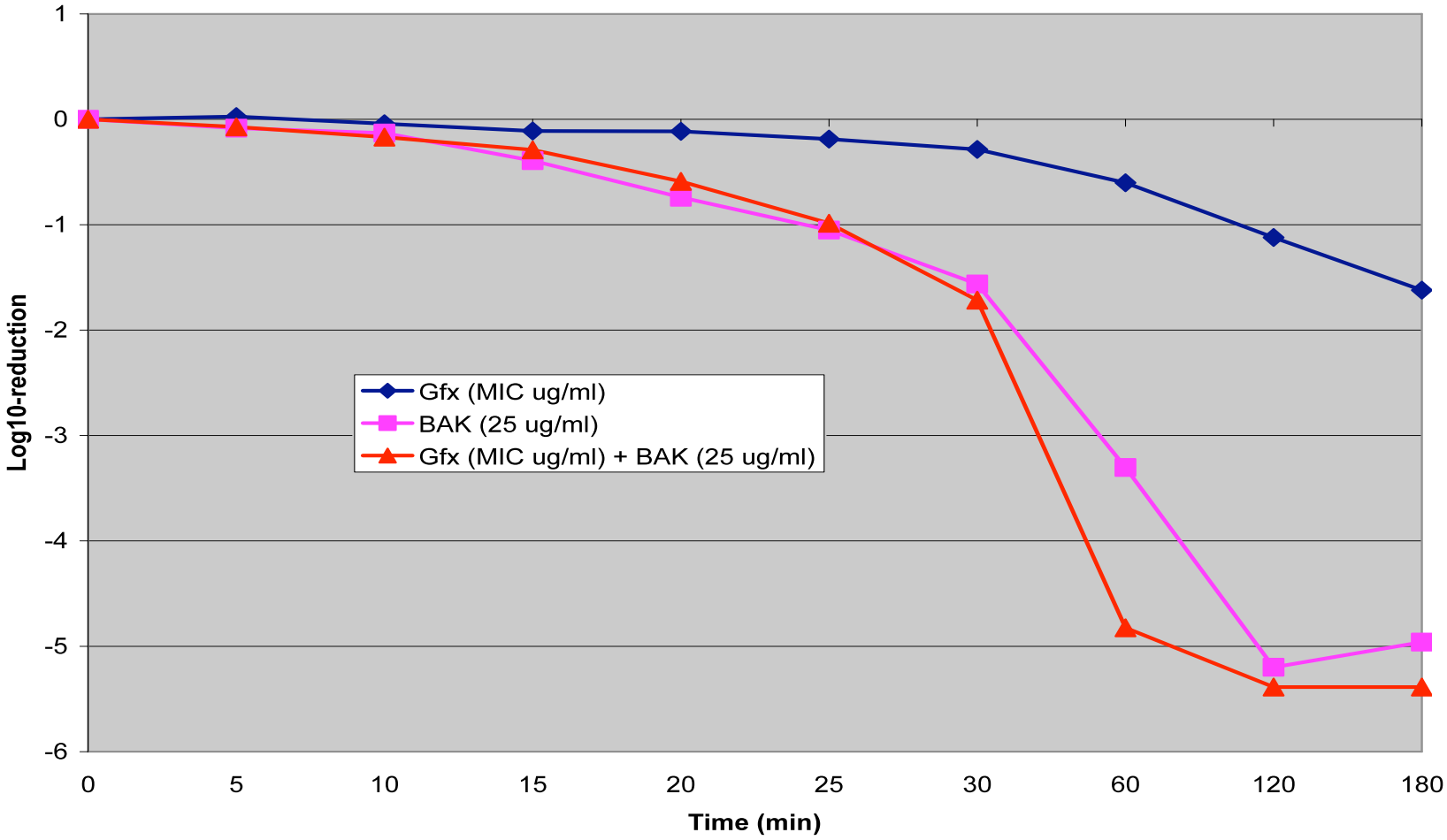


**Figure 3.3.3.3: $Log_{10}$-reduction of 2 clinical isolates of MRSA against gatifloxacin, BAK, and gatifloxacin plus BAK.**
Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

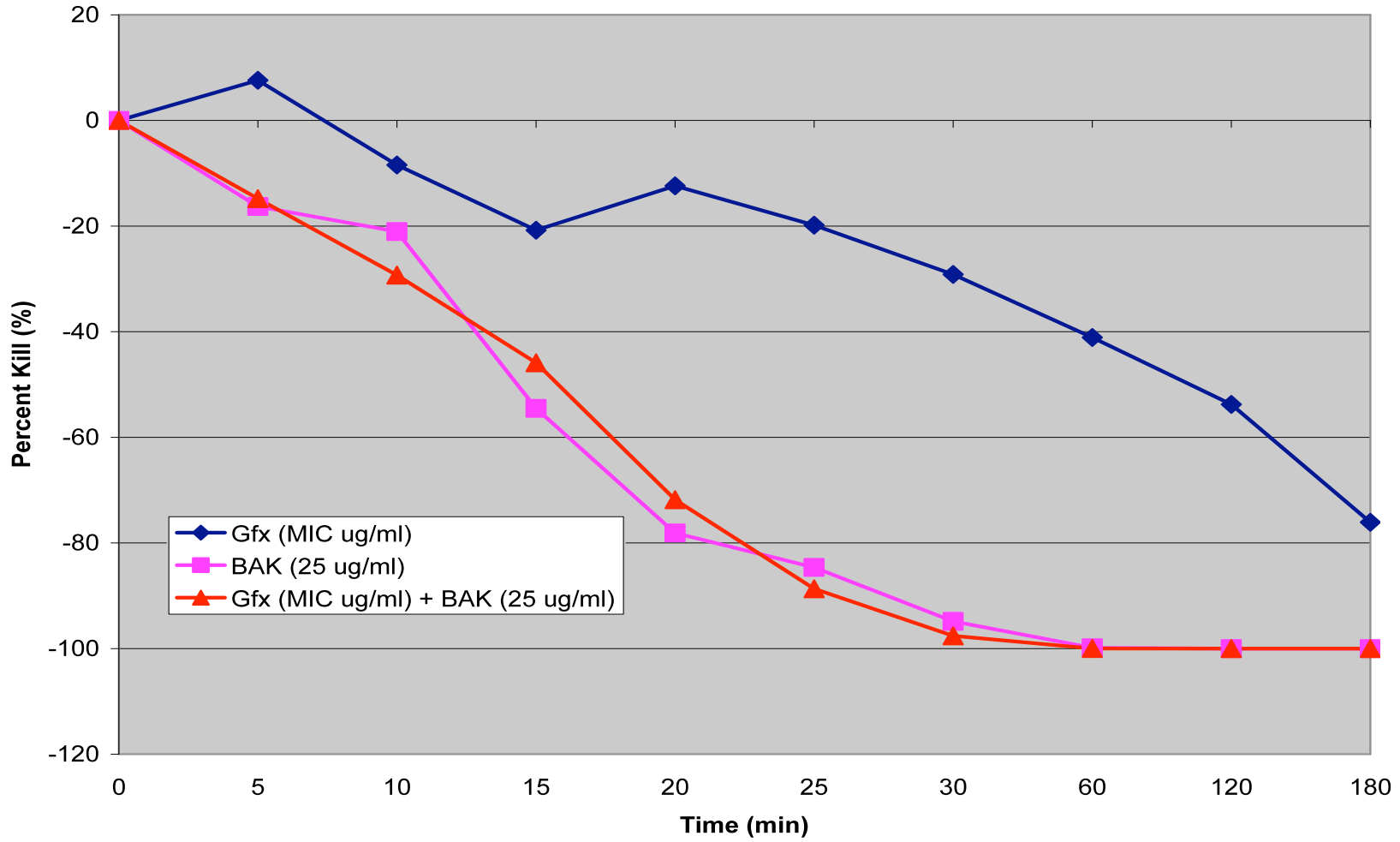


**Figure 3.3.3.4: Percent Kill (%) of 2 clinical isolates of MRSA against gatifloxacin, BAK, and gatifloxacin plus BAK.**
Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

activity of the commercial preparation Zymar® (0.3% gatifloxacin; 0.005% BAK) to the killing activity of gatifloxacin, BAK, and gatifloxacin plus BAK against clinical isolates of MRSA (n = 4). The concentration of gatifloxacin and BAK in Zymar® is 3,000 µg/ml and 50 µg/ml respectively. One drop of Zymar® has an approximate volume of 100µl. The amount of gatifloxacin and BAK in one drop or 100 µl of Zymar® is therefore approximately 300 µg and 5 µg respectively. To reflect the concentration of antimicrobial agent administered to the eye, the concentration of gatifloxacin and BAK used in the time-kill assays were 300 µg/ml and 5 µg/ml respectively. Very short time intervals were used in the time-kill studies with an ending time point of 15 minutes.

The MRSA isolates were grown to a bacterial density of $10^5$ CFU/ml and colony counts were taken at 0, 0.5, 1, 2, 3, 4, 5, 10, and 15 minutes after the addition of antimicrobial agent(s). The $\log_{10}$-reduction and percent kill of viable cells was calculated at each time point. Results from the four clinical MRSA isolates used in this time-kill assay were averaged. The results of the time-kill studies are summarized in Tables 3.3.3.5 and 3.3.3.6 and Figures 3.3.3.5 and 3.3.3.6. The $\log_{10}$-reduction (percent kill) of viable cells 15 minutes after the addition of gatifloxacin, BAK, gatifloxacin plus BAK, and Zymar® was -0.6 (-53.89%), -0.0 (-9.30%), -0.4 (-51.65%), and -0.8 (-59.09%) respectively. Zymar® had the best killing action ($\log_{10}$-reduction = -0.8) followed by gatifloxacin alone, gatifloxacin plus BAK, and BAK alone, however, it is evident from the time-kill graphs (Figures 3.3.3.5 and 3.3.3.6) that 15 minutes after the addition of antimicrobial agent to a bacterial population of MRSA, there is very poor killing activity exhibited by all the antimicrobial agents and combinations of antimicrobial agents tested. There was less than a 1 $\log_{10}$-reduction of viable cells 15 minutes after addition of

| Time (min) | $Log_{10}$-Reduction in Viable Cells | | | |
|---|---|---|---|---|
| | Gfx (300 µg/ml) | BAK (5 µg/ml) | Gfx (300 µg/ml) + BAK (5 µg/ml) | Zymar® (Gfx [300 µg/ml] + BAK [5 µg/ml])[a] |
| **0** | 0.000 | 0.000 | 0.000 | 0.000 |
| **0.5** | -0.163 | 0.035 | -0.193 | -0.130 |
| **1** | -0.149 | -0.027 | -0.264 | -0.223 |
| **2** | -0.194 | -0.044 | -0.175 | -0.250 |
| **3** | -0.176 | 0.039 | -0.287 | -0.189 |
| **4** | -0.283 | 0.015 | -0.270 | -0.188 |
| **5** | -0.273 | 0.001 | -0.295 | -0.332 |
| **10** | -0.428 | 0.009 | -0.416 | -0.429 |
| **15** | -0.591 | -0.043 | -0.417 | -0.834 |



**Table 3.3.3.5: $Log_{10}$-reduction of viable cells using gatifloxacin, BAK, gatifloxacin plus BAK, and Zymar® against 4 clinical isolates of MRSA.**

[a] Concentration of Gfx and BAK present in one drop (≈100 µl) of Zymar®

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

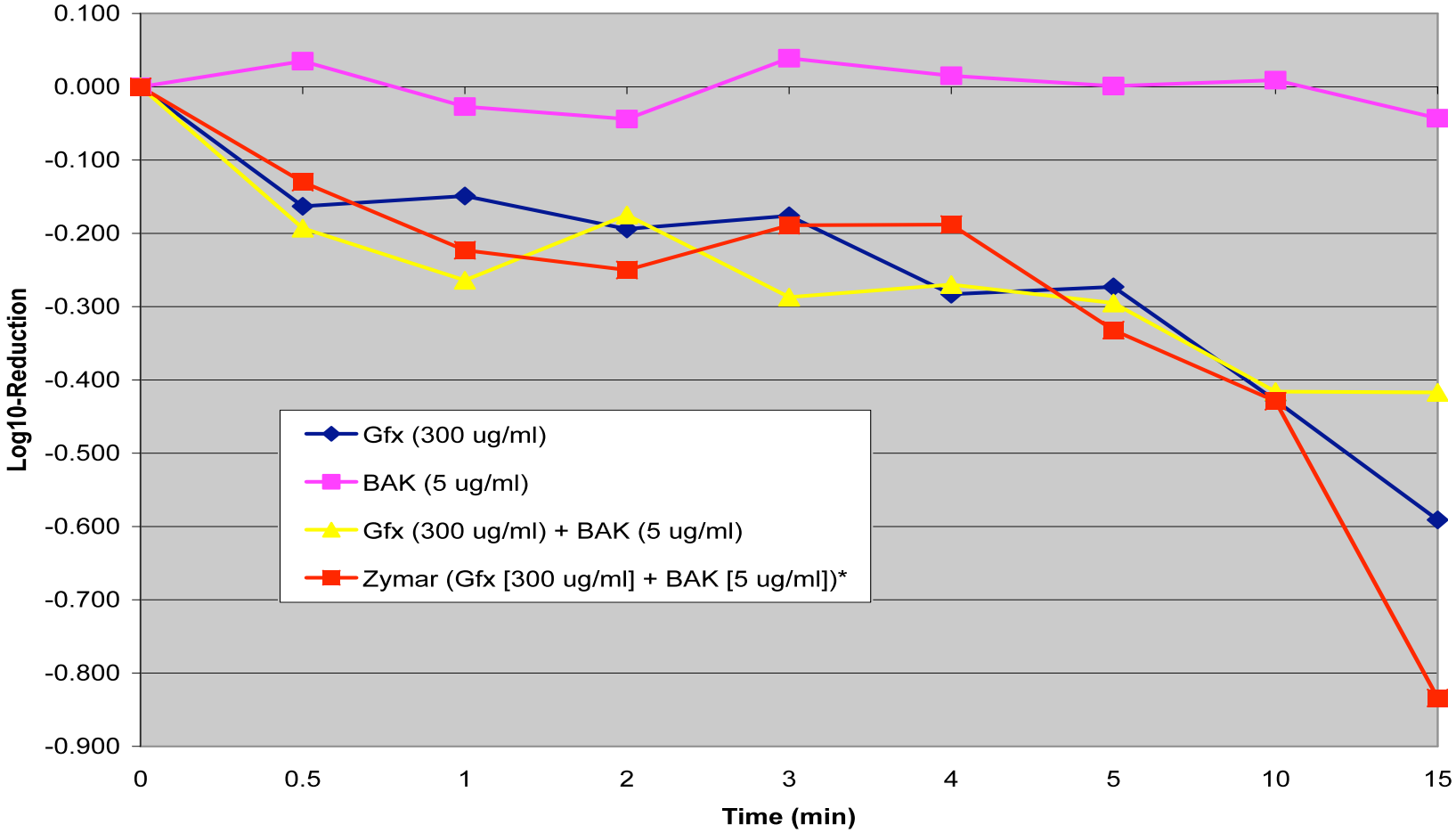


**Figure 3.3.3.5: $Log_{10}$-reduction of viable cells using gatifloxacin, BAK, gatifloxacin plus BAK, and Zymar® against 4 clinical isolates of MRSA.**

***** Concentration of Gfx and BAK present in one drop (≈ 100 µl) of Zymar®

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

| Time (min) | Percent Kill (%) | | | |
|---|---|---|---|---|
| | Gfx (300 µg/ml) | BAK (5 µg/ml) | Gfx (300 µg/ml) + BAK (5 µg/ml) | Zymar® (Gfx [300 µg/ml] + BAK [5 µg/ml])[a] |
| **0** | 0.000 | 0.000 | 0.000 | 0.000 |
| **0.5** | -29.276 | 9.870 | -34.127 | -24.313 |
| **1** | -27.118 | -5.698 | -40.837 | -38.020 |
| **2** | -34.270 | 11.372 | -31.547 | -42.286 |
| **3** | -31.803 | 9.551 | -45.902 | -33.269 |
| **4** | -41.943 | 4.682 | -44.301 | -33.400 |
| **5** | -41.475 | 1.243 | -45.735 | -48.905 |
| **10** | -49.344 | 3.649 | -54.862 | -54.541 |
| **15** | -53.885 | -9.295 | -51.651 | -59.086 |



**Table 3.3.3.6: Percent Kill (%) of viable cells using gatifloxacin, BAK, gatifloxacin plus BAK, and Zymar® against 4 clinical isolates of MRSA.**

[a] Concentration of Gfx and BAK present in one drop (≈ 100 µl) of Zymar®

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

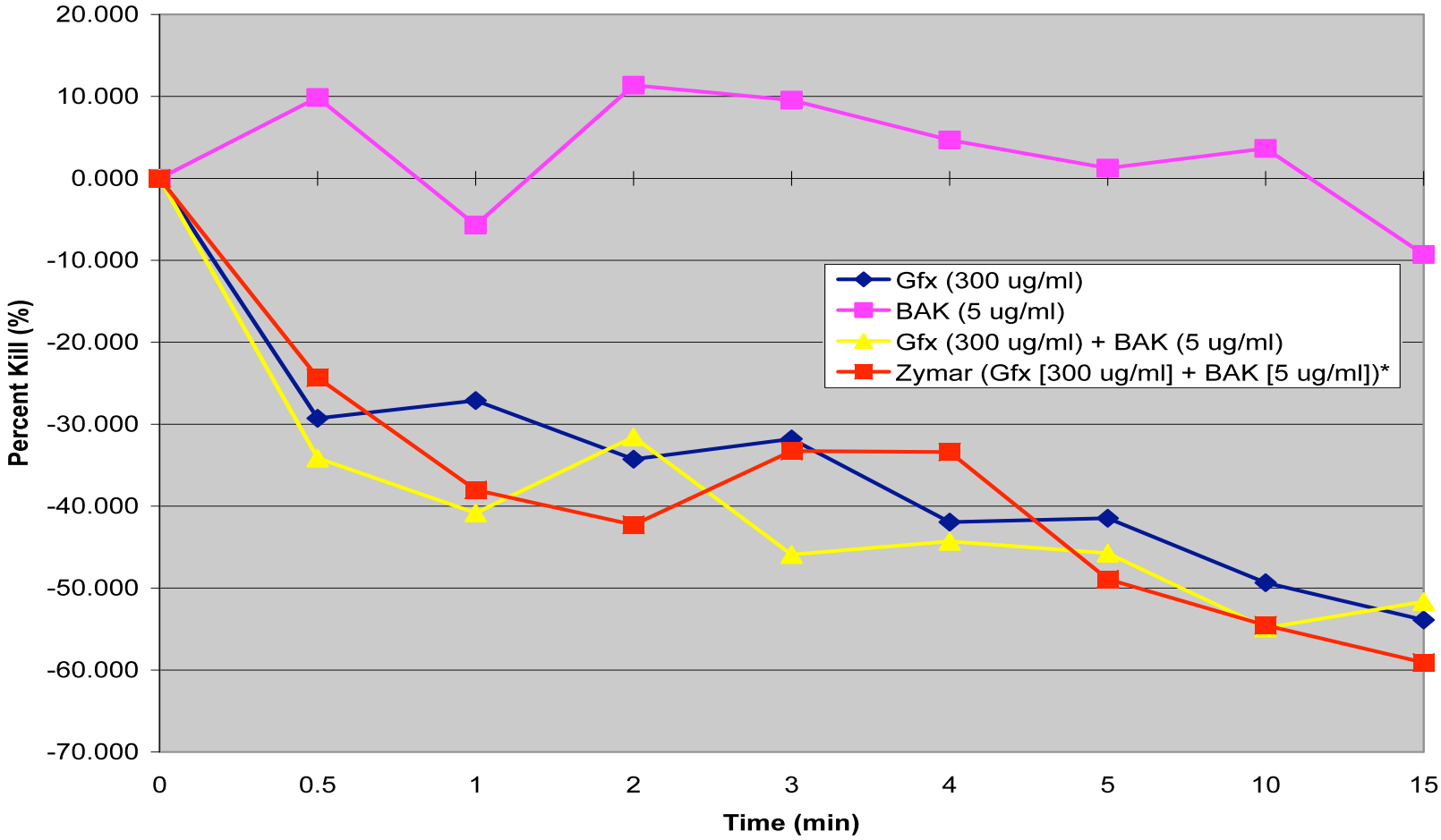


**Figure 3.3.3.6: Percent Kill (%) of viable cells using gatifloxacin, BAK, gatifloxacin plus BAK, and Zymar® against 4 clinical isolates of MRSA.**

***** Concentration of Gfx and BAK present in one drop (≈ 100 µl) of Zymar®

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

antimicrobial agent(s).

To investigate the effect of adding BAK at different time points during a time-kill study of gatifloxacin against clinical isolates of MRSA (n = 2), further conventional time-kill assays were performed. In these time-kill assays gatifloxacin, at a concentration corresponding to measured MIC values, was added at time 0 to a bacterial culture of MRSA at a bacterial load of $10^5$ CFU/ml. BAK at a concentration of 25 μg/ml was then added 5, 10, and 15 minutes after the addition of gatifloxacin. Colony counts were taken at 0, 5, 10, 15, 20, 25, 30, 60, 120, and 180 minutes after the initial addition of gatifloxacin and the $\log_{10}$-reduction and percent kill of viable cells was calculated. The results of these assays are found in Tables 3.3.3.7 and 3.3.3.8. The results are also visually represented in Figures 3.3.3.7 and 3.3.3.8.

The $\log_{10}$-reduction (percent kill) of viable cells 180 minutes after the addition of gatifloxacin was -1.6 (-76.08%), however when BAK was added 15, 10, and 5 minutes after the initial addition of gatifloxacin the $\log_{10}$-reduction (percent kill) of viable cells increased to -5.5 (-100%), -5.5 (-100%), and -5.6 (-100%) respectively. The addition of BAK enhanced the killing action of gatifloxacin against clinical isolates of MRSA (n = 2) and bactericidal activity (>3 $\log_{10}$-reduction) was observed at 180 minutes when gatifloxacin and BAK were used in conjunction, regardless of the time point BAK was added. In fact there was greater than a 3 $\log_{10}$-reduction of viable MRSA cells by 60 minutes when BAK was added 10 minutes ($\log_{10}$-reduction = -5.5) or 15 minutes ($\log_{10}$-reduction = -4.9) after gatifloxacin, and at 30 minutes when BAK was added 5 minutes ($\log_{10}$-reduction = -3.6) after gatifloxacin.

To determine whether the killing action of gatifloxacin plus BAK against clinical

| Time (min) | $Log_{10}$-Reduction of Viable Cells | | | | | |
|---|---|---|---|---|---|---|
| | **Gfx (MIC µg/ml)** | **BAK (25 µg/ml)** | **Gfx (MIC µg/ml) + BAK (25 µg/ml) at 15 minutes** | **Gfx (MIC µg/ml) + BAK (25 µg/ml) at 10 minutes** | **Gfx (MIC µg/ml) + BAK (25 µg/ml) at 5 minutes** | **Gfx (MIC µg/ml) + BAK (25 µg/ml) at 0 minutes** |
| **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **5** | 0.026 | -0.084 | -0.021 | 0.001 | -0.092 | -0.072 |
| **10** | -0.042 | -0.130 | -0.161 | -0.128 | -0.438 **(BAK 5 min)** | -0.169 |
| **15** | -0.111 | -0.392 | -0.277 | -0.470 **(BAK 5 min)** | -0.925 **(BAK 10 min)** | -0.290 |
| **20** | -0.114 | -0.740 | -0.905 **(BAK 5 min)** | -1.065 **(BAK 10 min)** | -1.249 **(BAK 15 min)** | -0.589 |
| **25** | -0.187 | -1.051 | -1.371 **(BAK 10 min)** | -1.784 **(BAK 15 min)** | -2.763 **(BAK 20 min)** | -0.986 |
| **30** | -0.286 | -1.563 | -2.174 **(BAK 15 min)** | -2.845 **(BAK 20 min)** | -3.590 **(BAK 25 min)** | -1.716 |
| **60** | -0.602 | -3.304 | -4.898 **(BAK 45 min)** | -5.509 **(BAK 50 min)** | -5.638 **(BAK 55 min)** | -4.825 |
| **120** | -1.121 | -5.199 | -5.460 **(BAK 105 min)** | -5.509 **(BAK 110 min)** | -5.638 **(BAK 115 min)** | -5.387 |
| **180** | -1.621 | -4.960 | -5.460 **(BAK 165 min)** | -5.509 **(BAK 170 min)** | -5.638 **(BAK 175 min)** | -5.387 |

**Table 3.3.3.7: $Log_{10}$-reduction of viable cells using gatifloxacin, BAK, and gatifloxacin plus BAK added at different time points against 2 clinical isolates of MRSA.**

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

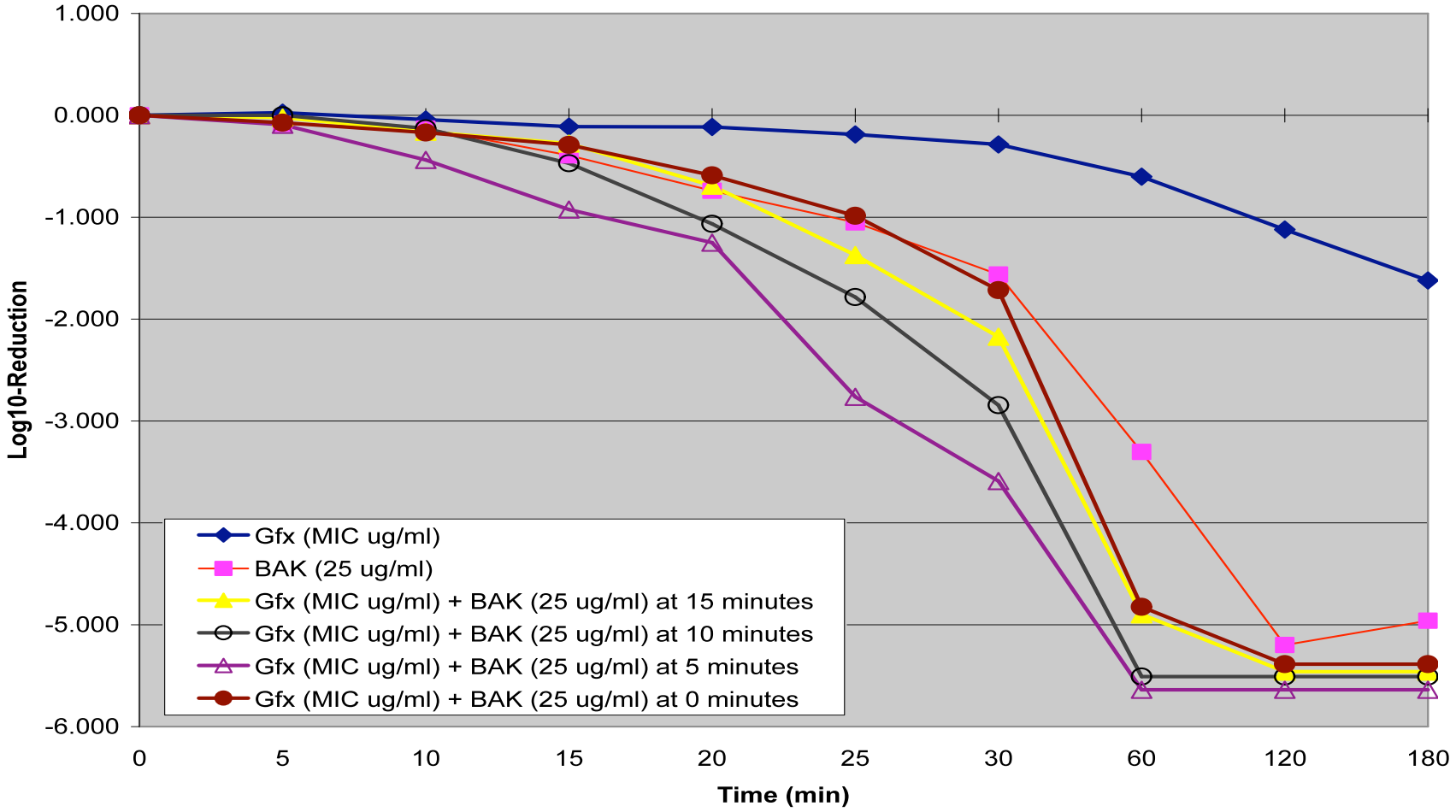


**Figure 3.3.3.7: $Log_{10}$-reduction of viable cells using gatifloxacin, BAK, and gatifloxacin plus BAK added at different time points against 2 clinical isolates of MRSA.**

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

| Time (min) | Percent Kill (%) | | | | | |
|---|---|---|---|---|---|---|
| | **Gfx (MIC µg/ml)** | **BAK (25 µg/ml)** | **Gfx (MIC µg/ml) + BAK (25 µg/ml) at 15 minutes** | **Gfx (MIC µg/ml) + BAK (25 µg/ml) at 10 minutes** | **Gfx (MIC µg/ml) + BAK (25 µg/ml) at 5 minutes** | **Gfx (MIC µg/ml) + BAK (25 µg/ml) at 0 minutes** |
| **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **5** | 7.614 | -16.299 | -4.395 | 3.383 | -18.540 | -14.793 |
| **10** | -8.426 | -21.029 | -30.656 | -25.367 | -63.382 **(BAK 5 min)** | -29.284 |
| **15** | -20.789 | -54.519 | -36.713 | -65.989 **(BAK 5 min)** | -87.564 **(BAK 10 min)** | -45.898 |
| **20** | -12.377 | -78.108 | -79.073 **(BAK 5 min)** | -91.316 **(BAK 10 min)** | -92.806 **(BAK 15 min)** | -71.825 |
| **25** | -19.826 | -84.648 | -95.459 **(BAK 10 min)** | -98.350 **(BAK 15 min)** | -99.808 **(BAK 20 min)** | -88.667 |
| **30** | -29.158 | -94.853 | -99.053 **(BAK 15 min)** | -99.852 **(BAK 20 min)** | -99.974 **(BAK 25 min)** | -97.597 |
| **60** | -41.098 | -99.860 | -99.998 **(BAK 45 min)** | -100.000 **(BAK 50 min)** | -100.000 **(BAK 55 min)** | -99.998 |
| **120** | -53.764 | -99.999 | -100.000 **(BAK 105 min)** | -100.000 **(BAK 110 min)** | -100.000 **(BAK 115 min)** | -100.000 |
| **180** | -76.079 | -99.999 | -100.000 **(BAK 165 min)** | -100.000 **(BAK 170 min)** | -100.000 **(BAK 175 min)** | -100.000 |

**Table 3.3.3.8: Percent Kill (%) of viable cells using gatifloxacin, BAK, and gatifloxacin plus BAK added at different time points against 2 clinical isolates of MRSA.**

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

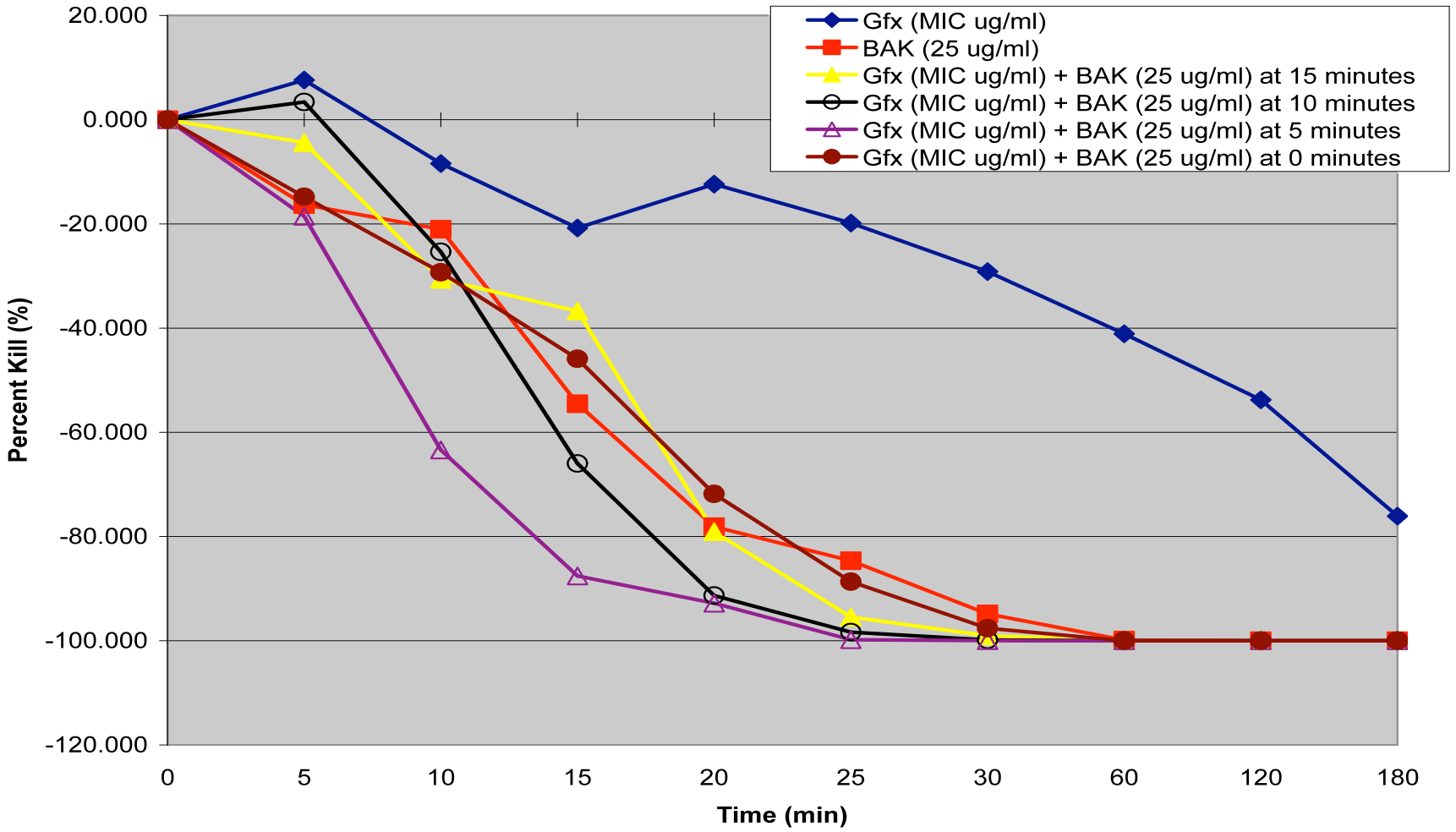


**Figure 3.3.3.8: Percent Kill (%) of viable cells using gatifloxacin, BAK, and gatifloxacin plus BAK added at different time points against 2 clinical isolates of MRSA.**

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

isolates of MRSA could be increased by first treating bacterial colonies with BAK and then adding gatifloxacin at different time points, conventional time-kill assays were performed. These time-kill assays were an exact reversal of the previous time-kill assays. BAK, at a concentration of 25 μg/ml, was added at time 0 to a bacterial culture of MRSA at a bacterial load of $10^5$ CFUs. Gatifloxacin, at a concentration corresponding to measured MIC values, was then added 5, 10, and 15 minutes after the addition of BAK. Colony counts were taken at 0, 5, 10, 15, 20, 25, 30, 60, 120, and 180 minutes after the initial addition of BAK and the $\log_{10}$-reduction and percent kill of viable cells was calculated. The results of these assays are found in Tables 3.3.3.9 and 3.3.3.10. The results are also visually represented in Figures 3.3.3.9 and 3.3.3.10.

The $\log_{10}$-reduction (percent kill) of viable cells 180 minutes after the addition of BAK was -5.0 (99.99%). The addition of gatifloxacin 15, 10, and 5 minutes after the initial addition of BAK resulted in $\log_{10}$-reduction (percent kill) values of -5.4 (-100%), -5.5 (-100%), and -5.5 (-100%) respectively 180 minutes after the initial addition of BAK. It is evident from the kill curves (Figures 3.3.3.9 and 3.3.3.10) that the addition of gatifloxacin had minimal impact on the killing action of BAK.

**3.4 Susceptibility Testing of Alexidine**

Alexidine is a cationic antimicrobial agent belonging to the bisbiguanide family. It has been used as an effective disinfectant in the dental industry for decades and is currently being looked at as a potential antimicrobial agent for ocular infections. We completed MIC and MPC testing with alexidine against Gram-positive and Gram-negative organisms often associated with ocular infection, including MRSA, MSSA, *S. pneumoniae*, *P. aeruginosa*, and *Haemophilus influenzae*. We also looked at the killing

| Time (min) | $Log_{10}$-Reduction of Viable Cells | | | | | |
|---|---|---|---|---|---|---|
| | **BAK (25 µg/ml)** | **Gfx (MIC µg/ml)** | **BAK (25 µg/ml) + Gfx (MIC µg/ml) at 15 minutes** | **BAK (25 µg/ml) + Gfx (MIC µg/ml) at 10 minutes** | **BAK (25 µg/ml) + Gfx (MIC µg/ml) at 5 minutes** | **BAK (25 µg/ml) + Gfx (MIC µg/ml) at 0 minutes** |
| **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **5** | -0.084 | 0.026 | 0.003 | -0.149 | -0.211 | -0.072 |
| **10** | -0.130 | -0.042 | -0.294 | -0.240 | -0.419 **(Gfx 5 min)** | -0.169 |
| **15** | -0.392 | -0.111 | -0.611 | -0.601 **(Gfx 5 min)** | -0.837 **(Gfx 10 min)** | -0.290 |
| **20** | -0.740 | -0.114 | -0.905 **(Gfx 5 min)** | -0.937 **(Gfx 10 min)** | -1.400 **(Gfx 15 min)** | -0.589 |
| **25** | -1.051 | -0.187 | -1.305 **(Gfx 10 min)** | -1.590 **(Gfx 15 min)** | -2.132 **(Gfx 20 min)** | -0.986 |
| **30** | -1.563 | -0.286 | -1.929 **(Gfx 15 min)** | -2.163 **(Gfx 20 min)** | -3.041 **(Gfx 25 min)** | -1.716 |
| **60** | -3.304 | -0.602 | -4.350 **(Gfx 45 min)** | -5.242 **(Gfx 50 min)** | -5.274 **(Gfx 55 min)** | -4.825 |
| **120** | -5.199 | -1.121 | -5.423 **(Gfx 105 min)** | -5.503 **(Gfx 110 min)** | -5.535 **(Gfx 115 min)** | -5.387 |
| **180** | -4.960 | -1.621 | -5.423 **(Gfx 165 min)** | -5.503 **(Gfx 170 min)** | -5.535 **(Gfx 175 min)** | -5.387 |

**Table 3.3.3.9: $Log_{10}$-reduction of viable cells using BAK, gatifloxacin, and BAK plus gatifloxacin added at different time points against 2 clinical isolates of MRSA.**

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

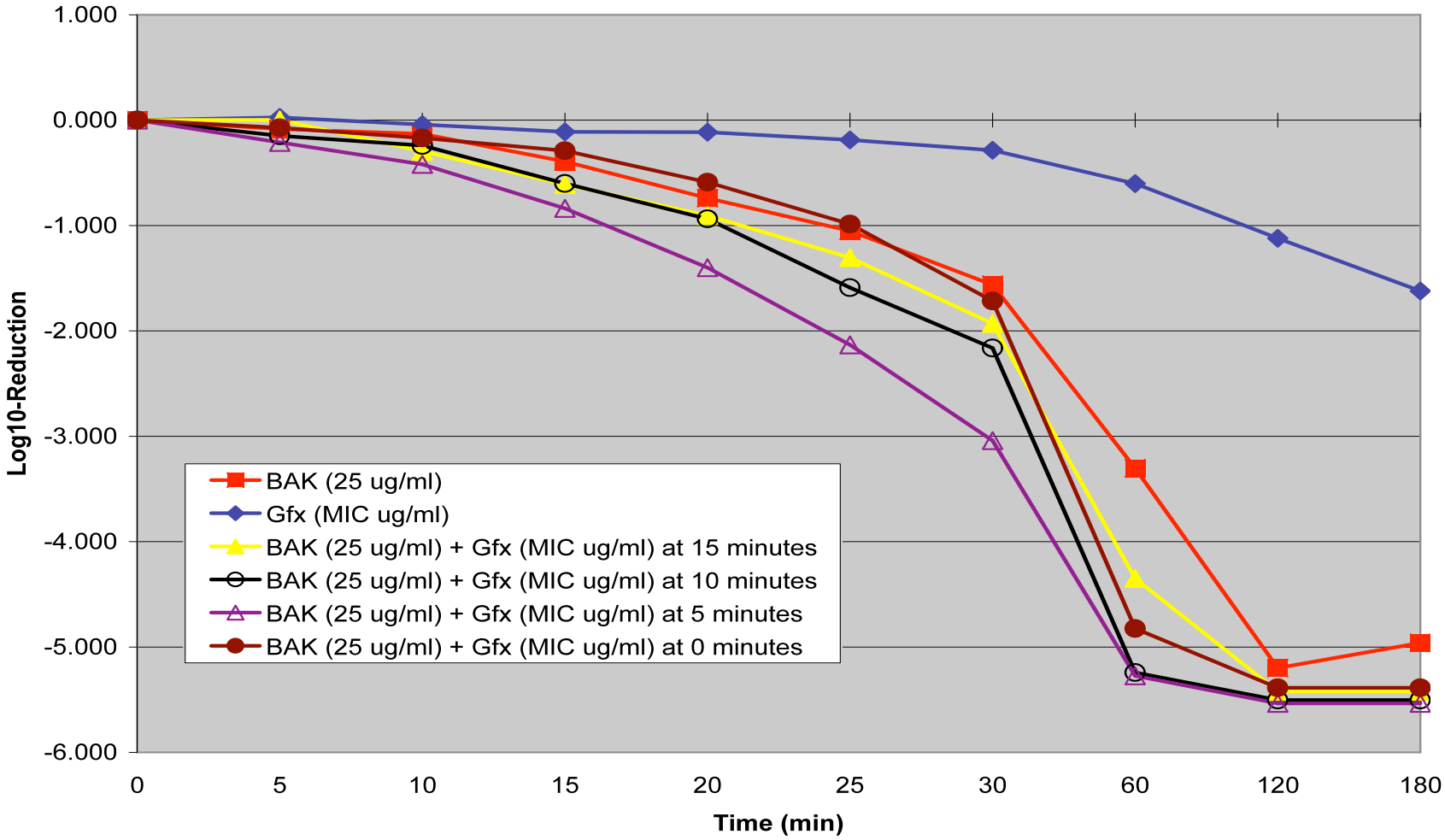


**Figure 3.3.3.9: $Log_{10}$-reduction of viable cells using BAK, gatifloxacin, and BAK plus gatifloxacin added at different time points against 2 clinical isolates of MRSA.**

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

| Time (min) | Percent Kill (%) | | | | | |
|---|---|---|---|---|---|---|
| | **BAK (25 µg/ml)** | **Gfx (MIC µg/ml)** | **BAK (25 µg/ml) + Gfx (MIC µg/ml) at 15 minutes** | **BAK (25 µg/ml) + Gfx (MIC µg/ml) at 10 minutes** | **BAK (25 µg/ml) + Gfx (MIC µg/ml) at 5 minutes** | **BAK (25 µg/ml) + Gfx (MIC µg/ml) at 0 minutes** |
| **0** | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| **5** | -16.299 | 7.614 | 1.412 | -24.778 | -37.831 | -14.793 |
| **10** | -21.029 | -8.426 | -47.089 | -41.261 | -60.241 **(BAK 5 min)** | -29.284 |
| **15** | -54.519 | -20.789 | -74.028 | -72.444 **(BAK 5 min)** | -83.881 **(BAK 10 min)** | -45.898 |
| **20** | -78.108 | -12.377 | -85.633 **(BAK 5 min)** | -88.886 **(BAK 10 min)** | -94.978 **(BAK 15 min)** | -71.825 |
| **25** | -84.648 | -19.826 | -93.042 **(BAK 10 min)** | -95.285 **(BAK 15 min)** | -99.114 **(BAK 20 min)** | -88.667 |
| **30** | -94.853 | -29.158 | -98.372 **(BAK 15 min)** | -98.645 **(BAK 20 min)** | -99.846 **(BAK 25 min)** | -97.597 |
| **60** | -99.860 | -41.098 | -99.994 **(BAK 45 min)** | -100.000 **(BAK 50 min)** | -100.000 **(BAK 55 min)** | -99.998 |
| **120** | -99.999 | -53.764 | -100.000 **(BAK 105 min)** | -100.000 **(BAK 110 min)** | -100.000 **(BAK 115 min)** | -100.000 |
| **180** | -99.999 | -76.079 | -100.000 **(BAK 165 min)** | -100.000 **(BAK 170 min)** | -100.000 **(BAK 175 min)** | -100.000 |

**Table 3.3.3.10: Percent Kill (%) of viable cells using BAK, gatifloxacin, and BAK plus gatifloxacin added at different time points against 2 clinical isolates of MRSA.**

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

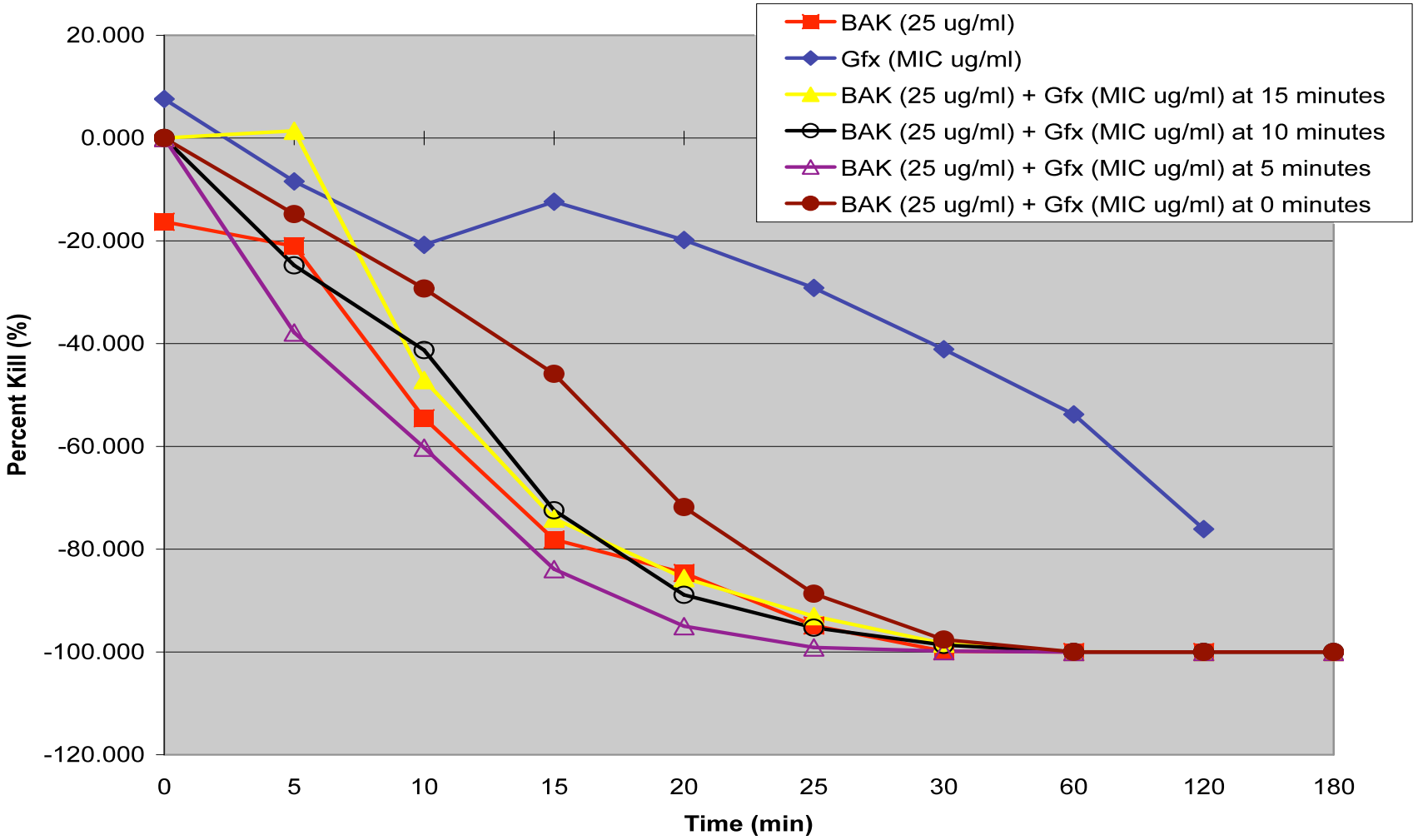


**Figure 3.3.3.10: Percent Kill (%) of viable cells using BAK, gatifloxacin, and BAK plus gatifloxacin added at different time points against 2 clinical isolates of MRSA.**

Gfx = gatifloxacin; BAK = Benzalkonium chloride; MRSA = Methicillin-resistant *Staphylococcus aureus*

activity of Alexidine against these pathogens.

### 3.4.1 Comparative MIC Results

Minimum inhibitory concentration (MIC) values were measured for clinical isolates of MRSA, MSSA, *S. pneumoniae*, *P. aeruginosa*, and *H. influenzae* against alexidine, levofloxacin, gatifloxacin, and moxifloxacin. Using CLSI recommended procedures, $MIC_{50}$ and $MIC_{90}$ values were measured for each pathogen and antimicrobial agent [65]. The resulting $MIC_{50}$ and $MIC_{90}$ values are shown in Table 3.4.1.1.

Measured $MIC_{50}$ values (μg/ml) for alexidine, gatifloxacin, moxifloxacin, and levofloxacin against MRSA were 1, >2, 1, and 4 respectively; against MSSA 1, 0.063, 0.031, and 0.125 respectively; against *S. pneumoniae* 0.5, 0.25, 0.125, and 0.5 respectively; against *H. influenzae* 4, 0.016, 0.016, and 0.016 respectively; and against *P. aeruginosa* 4, 1, 1, and 1 respectively. Measured $MIC_{90}$ values (μg/ml) for alexidine, gatifloxacin, moxifloxacin, and levofloxacin against MRSA were 2, >2, 4, and >8 respectively; against MSSA 2, 2, 1, and 4 respectively; against *S. pneumoniae* 0.5, 0.25, 0.125, and 1 respectively; against *H. influenzae* 8, 0.031, 0.031, and 0.031 respectively; and against *P. aeruginosa* 4, 4, 8, and 4 respectively. The results indicate that alexidine is more active *in vitro* (lower MIC values) against Gram-positive pathogens than against Gram-negative pathogens. It is also evident that the fluoroquinolones (gatifloxacin, moxifloxacin, and levofloxacin) are more effective antimicrobial agents *in vitro* (lower MIC values) against clinical isolates of *H. influenzae* than alexidine.

### 3.4.2 MPC Results

Mutant prevention concentration (MPC) values were measured for alexidine against clinical isolates of MRSA, MSSA, and *S. pneumoniae*.

| | Organism | n | $MIC_{50}$[a] (µg/ml) | $MIC_{90}$[b] (µg/ml) |
|---|---|---|---|---|
| **Alexidine** | MRSA | 83 | 1 | 2 |
| | MSSA | 24 | 1 | 2 |
| | *S. pneumoniae* | 47 | 0.5 | 0.5 |
| | *H. influenzae* | 41 | 4 | 8 |
| | *P. aeruginosa* | 22 | 4 | 4 |
| **Gatifloxacin** | MRSA | 72 | >2 | >2 |
| | MSSA | 24 | 0.063 | 2 |
| | *S. pneumoniae* | 47 | 0.25 | 0.25 |
| | *H. influenzae* | 29 | 0.016 | 0.031 |
| | *P. aeruginosa* | 22 | 1 | 4 |
| **Moxifloxacin** | MRSA | 83 | 1 | 4 |
| | MSSA | 24 | 0.031 | 1 |
| | *S. pneumoniae* | 48 | 0.125 | 0.125 |
| | *H. influenzae* | 27 | 0.016 | 0.031 |
| | *P. aeruginosa* | 22 | 1 | 8 |
| **Levofloxacin** | MRSA | 83 | 4 | >8 |
| | MSSA | 24 | 0.125 | 4 |
| | *S. pneumoniae* | 48 | 0.5 | 1 |
| | *H. influenzae* | 29 | 0.016 | 0.031 |
| | *P. aeruginosa* | 22 | 1 | 4 |

**Table 3.4.1.1: Comparative $MIC_{50}$ and $MIC_{90}$ values for alexidine, gatifloxacin, moxifloxacin, and levofloxacin against Gram-positive and Gram-negative organisms.**

[a] The drug concentration inhibiting 50% of isolates tested

[b] The drug concentration inhibiting 90% of isolates tested

MIC = minimum inhibitory concentration; MRSA = Methicillin-resistant *Staphylococcus aureus*; MSSA = Methicillin-susceptible *Staphylococcus aureus*

#### 3.4.2.1 Traditional Agar Dilution Method

Minimum inhibitory concentration (MIC) values and MPC values were measured for alexidine against clinical isolates of *S. pneumoniae* (n = 47). MIC testing was performed using CLSI recommended guidelines and MPC testing was performed using the traditional agar dilution method (Section 2.3.2) [85]. The $MIC_{50}$, $MIC_{90}$, and MIC range values (μg/ml) were 0.5, 0.5, and 0.25 – 1 respectively. The $MPC_{50}$, $MPC_{90}$, and MPC range values (μg/ml) were >16, >16, and 8 - >16 respectively. The $MPC_{50}$ and $MPC_{90}$ values are greater than 32-fold higher than the $MIC_{90}$ values measured for the same *S. pneumoniae* isolates.

Mutant prevention concentration (MPC) testing of alexidine against MRSA and MSSA was attempted several times using the traditional established methodology (Section 2.3.2). Tryptic soy agar plates containing 2-fold concentration increments of alexidine were created and MRSA isolates were inoculated at a density of $\geq 10^9$ CFU/ml onto these plates. Alexidine concentrations (μg/ml) of 1, 2, 4, 8, 16, and 32 were used. Unfortunately, each experimental attempt yielded non-reproducible results. In general there was confluent bacterial growth on all drug dilution plates up to those containing 2 μg/ml of alexidine. On plates containing 4 μg/ml, 8 μg/ml, 16 μg/ml, and 32 μg/ml of alexidine, random single colonies were observed. The single colonies would often be present on a drug plate with a concentration of 32 μg/ml but not on drug plates containing 16 μg/ml or 8 μg/ml. This "skipped well" phenomenon was observed for several isolates and when the experiment was repeated the results would be different (i.e. different wells would be skipped).

In one MPC assay using alexidine against clinical MRSA isolates (n = 56) the number of isolates growing at each drug concentration was recorded (Table 3.4.2.1.1). The number of isolates exhibiting the "skipped well" phenomenon were also recorded (Table 3.4.2.1.2). Twenty-two MRSA isolates skipped wells covering a 4-fold difference in alexidine concentration, 3 MRSA isolates skipped wells covering an 8-fold difference in alexidine concentration, and 5 MRSA isolates skipped wells covering a 16-fold difference in alexidine concentration. There was also one clinical MRSA isolate that skipped wells covering a 32-fold difference in alexidine concentration. Nineteen MRSA isolates exhibiting skipped well phenomenon were back-tested and shown to have the same MIC values as experimentally determined prior to MPC testing.

#### 3.4.2.2 Modified Microbroth Dilution Method

The modified microbroth dilution method for determining MPC values was used to determine the MPC values of alexidine against clinical isolates of MRSA and MSSA. The modified MPC method was used because the traditional MPC method generated non-reproducible results (Section 3.4.2.1). The modified MPC values for alexidine against clinical isolates of MRSA (n = 3) and MSSA (ATCC 29213) were both >64 μg/ml. Colony counts were performed to ensure that the bacterial density in the 96-well microtitre panel was $\geq 10^9$ CFU/ml. The measured MPC values are greater than 32-fold higher than the measure MIC values for alexidine against MRSA and MSSA.

### 3.4.3 Time-Kill Results

To determine the killing action of alexidine, conventional time-kill studies were completed with alexidine against clinical isolates of MRSA (n=3), MSSA (n = 1), *S. pneumoniae* (n = 3), and *P. aeruginosa* (n = 2). The killing action of alexidine was

| Alexidine concentration (µg/ml) | # Isolates Growing |
|---|---|
| 1 | 56 |
| 2 | 43 |
| 4 | 34 |
| 8 | 25 |
| 16 | 20 |
| 32 | 16 |

**Table 3.4.2.1.1: The number of MRSA isolates (n = 56) growing at different concentrations of alexidine.**
MRSA = Methicillin-resistant *Staphylococcus aureus*



| Skipped Well Dilution | # of Isolates |
|---|---|
| 4-fold | 22 |
| 8-fold | 3 |
| 16-fold | 5 |
| 32-fold | 1 |

**Table 3.4.2.1.2: The number of MRSA isolates exhibiting various degrees of "skipped well" phenomenon during traditional MPC testing.**
MRSA = Methicillin-resistant *Staphylococcus aureus*

measured using three different drug concentrations corresponding to the MIC (μg/ml), 2 x MIC (μg/ml), and 4 x MIC (μg/ml). The bacterial isolates were grown to a bacterial density of $10^5$ CFU/ml and colony counts were taken, in triplicate, at 0, 15, 30, 45, 60, 75, 90, 120, and 180 minutes after the addition of alexidine. The $\log_{10}$-reduction and percent kill of viable cells was calculated at each time point.

In kill-studies using alexidine against clinical isolates of MRSA (n = 3) there was a greater than 3 $\log_{10}$-reduction of viable cells 180 minutes after the addition of alexidine when concentrations corresponding to the MIC, 2 x MIC, and 4 x MIC were used (Table 3.4.3.1 and Figure 3.4.3.1). In fact, there was a greater than 3 $\log_{10}$-reduction at 30 minutes when a concentration of 2 x MIC was used and at 15 minutes when a concentration of 4 x MIC was used (Table 3.4.3.1 and Figure 3.4.3.1). Using an alexidine concentration of the MIC against clinical isolates of MRSA, there was 99.99% kill of bacterial cells 90 minutes after the addition of alexidine (Table 3.4.3.2 and Figure 3.4.3.2). With an alexidine concentration corresponding to 2 x MIC and 4 x MIC was used against clinical isolates of MRSA there was a 100% kill of bacterial cells 45 minutes and 30 minutes after the addition of alexidine respectively (Table 3.4.3.2 and Figure 3.4.3.2).

In kill-studies using alexidine against a clinical isolate of MSSA there was a greater than 3 $\log_{10}$-reduction of viable cells 45 minutes after the addition of alexidine when concentrations corresponding to the MIC, 2 x MIC, and 4 x MIC were used (Table 3.4.3.3 and Figure 3.4.3.3). The kill-pattern was similar to that observed with alexidine against MRSA with a greater than 3 $\log_{10}$-reduction in viable cells calculated at 30 minutes when a concentration of 2 x MIC was used and at 15 minutes when a

concentration of 4 x MIC was used (Table 3.4.3.3 and Figure 3.4.3.3). At 180 minutes, 99.99% of all viable cells were killed using an alexidine concentration of the MIC, while 100% of all viable cells were killed using concentrations of 2 x MIC and 4 x MIC (Table 3.4.3.4 and Figure 3.4.3.4).

In kill-studies using alexidine against clinical isolates of *S. pneumoniae* (n = 3) there was a greater than 4 $\log_{10}$-reduction of viable cells 30 minutes after the addition of alexidine when concentrations of the MIC, 2 x MIC, and 4 x MIC were used (Table 3.4.3.5 and Figure 3.4.3.5). In fact, there was 100% kill of all viable cells at 60 minutes, 30 minutes, and 15 minutes when alexidine concentrations corresponding to the MIC, 2 x MIC, and 4 x MIC were used respectively against clinical isolates of *S. pneumoniae* (Table 3.4.3.6 and Figure 3.4.3.6).

Alexidine displayed perhaps the fastest killing action against clinical isolates of *P. aeruginosa* (n = 2). There was a greater than 4 $\log_{10}$-reduction of viable cells 15 minutes after the addition of alexidine at concentrations corresponding to the MIC, 2 x MIC, and 4 x MIC (Table 3.4.3.7 and Figure 3.4.3.7). Furthermore, there was a calculated percent kill of 100% at 30 minutes after the addition of alexidine when a concentration of the MIC was used against clinical isolates of *P. aeruginosa*. There was 100% kill of viable cells calculated just 15 minutes after the addition of alexidine when concentrations of 2 x MIC and 4 x MIC were used against clinical isolates of *P. aeruginosa* (Table 3.4.3.8 and Figure 3.4.3.8). Alexidine was a potent killer of both Gram-positive and Gram-negative organisms; we calculated >99.99% kill of viable cells within 15 minutes using alexidine concentrations corresponding to 2 x MIC and 4 x MIC against all clinical isolates tested (MRSA, MSSA, *S. pneumoniae*, and *P. aeruginosa*).

| Time (min) | $Log_{10}$-Reduction of Viable Cells | | |
|---|---|---|---|
| | Alexidine Concentration | | |
| | MIC (µg/ml) | 2 x MIC (µg/ml) | 4 x MIC (µg/ml) |
| **0** | 0.000 | 0.000 | 0.000 |
| **15** | -0.663 | -2.191 | -3.937 |
| **30** | -0.978 | -3.460 | -5.087 |
| **45** | -1.548 | -4.122 | -5.087 |
| **60** | -1.885 | -4.170 | -5.087 |
| **75** | -2.049 | -5.085 | -5.087 |
| **90** | -2.404 | -5.260 | -5.087 |
| **120** | -2.810 | -5.260 | -5.087 |
| **180** | -3.619 | -5.260 | -5.087 |

**Table 3.4.3.1: The $Log_{10}$-reduction of viable cells using different concentrations of alexidine against clinical isolates of MRSA (n = 3)**
MIC = minimum inhibitory concentration; MRSA = Methicillin-resistant *Staphylococcus aureus*



| Time (min) | Percent Kill (%) | | |
|---|---|---|---|
| | Alexidine Concentration | | |
| | MIC (µg/ml) | 2 x MIC (µg/ml) | 4 x MIC (µg/ml) |
| **0** | 0.000 | 0.000 | 0.000 |
| **15** | -95.750 | -99.753 | -99.994 |
| **30** | -99.728 | -99.988 | -100.000 |
| **45** | -99.986 | -100.000 | -100.000 |
| **60** | -99.997 | -100.000 | -100.000 |
| **75** | -99.998 | -100.000 | -100.000 |
| **90** | -99.999 | -100.000 | -100.000 |
| **120** | -99.999 | -100.000 | -100.000 |
| **180** | -99.999 | -100.000 | -100.000 |

**Table 3.4.3.2: The Percent Kill (%) of viable cells using different concentrations of alexidine against clinical isolates of MRSA (n = 3)**
MIC = minimum inhibitory concentration; MRSA = Methicillin-resistant *Staphylococcus aureus*

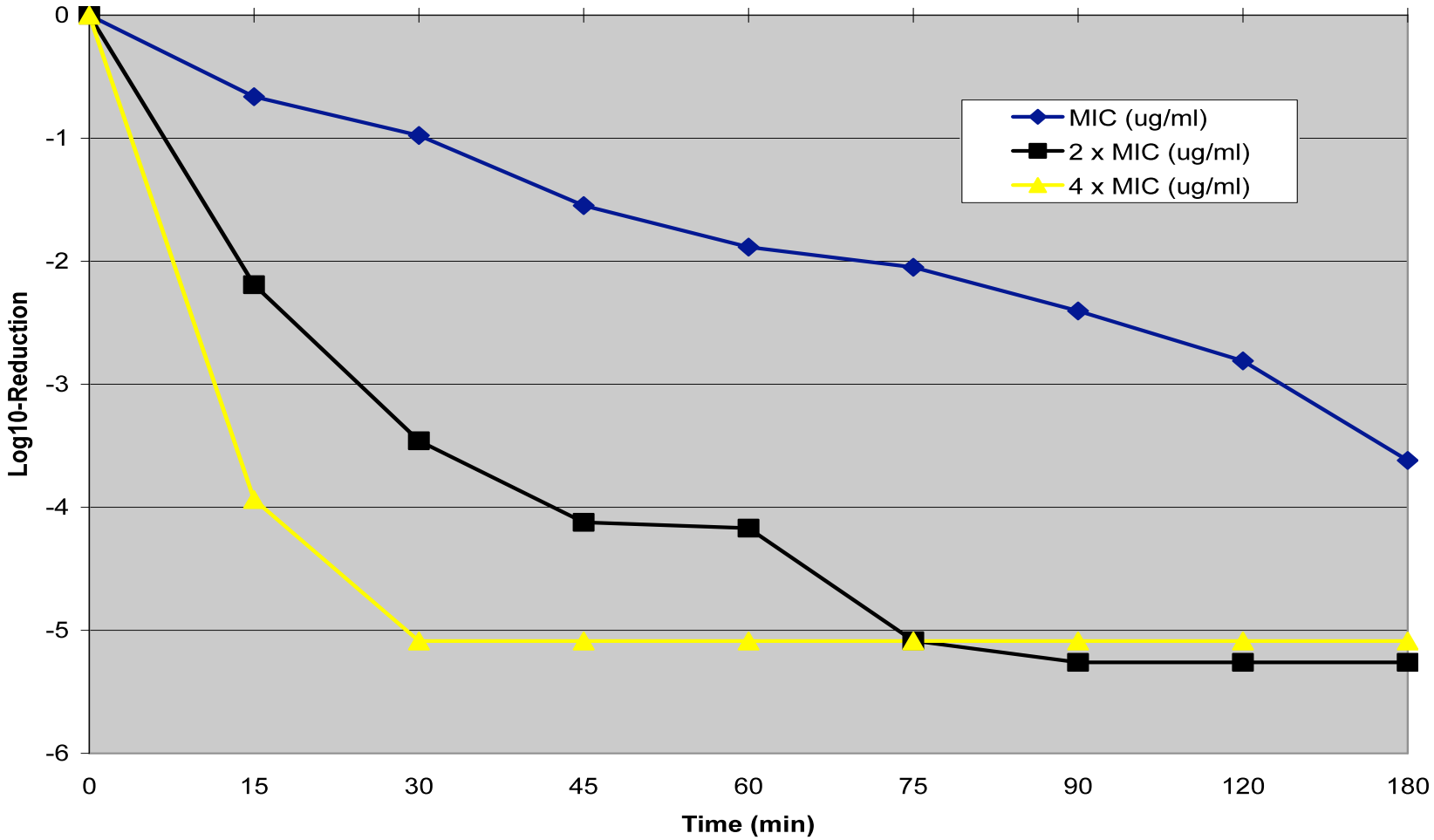


**Figure 3.4.3.1: The $Log_{10}$-reduction of viable cells using different concentrations of alexidine against clinical isolates of MRSA (n = 3)**
MIC = minimum inhibitory concentration; MRSA = Methicillin-resistant *Staphylococcus aureus*

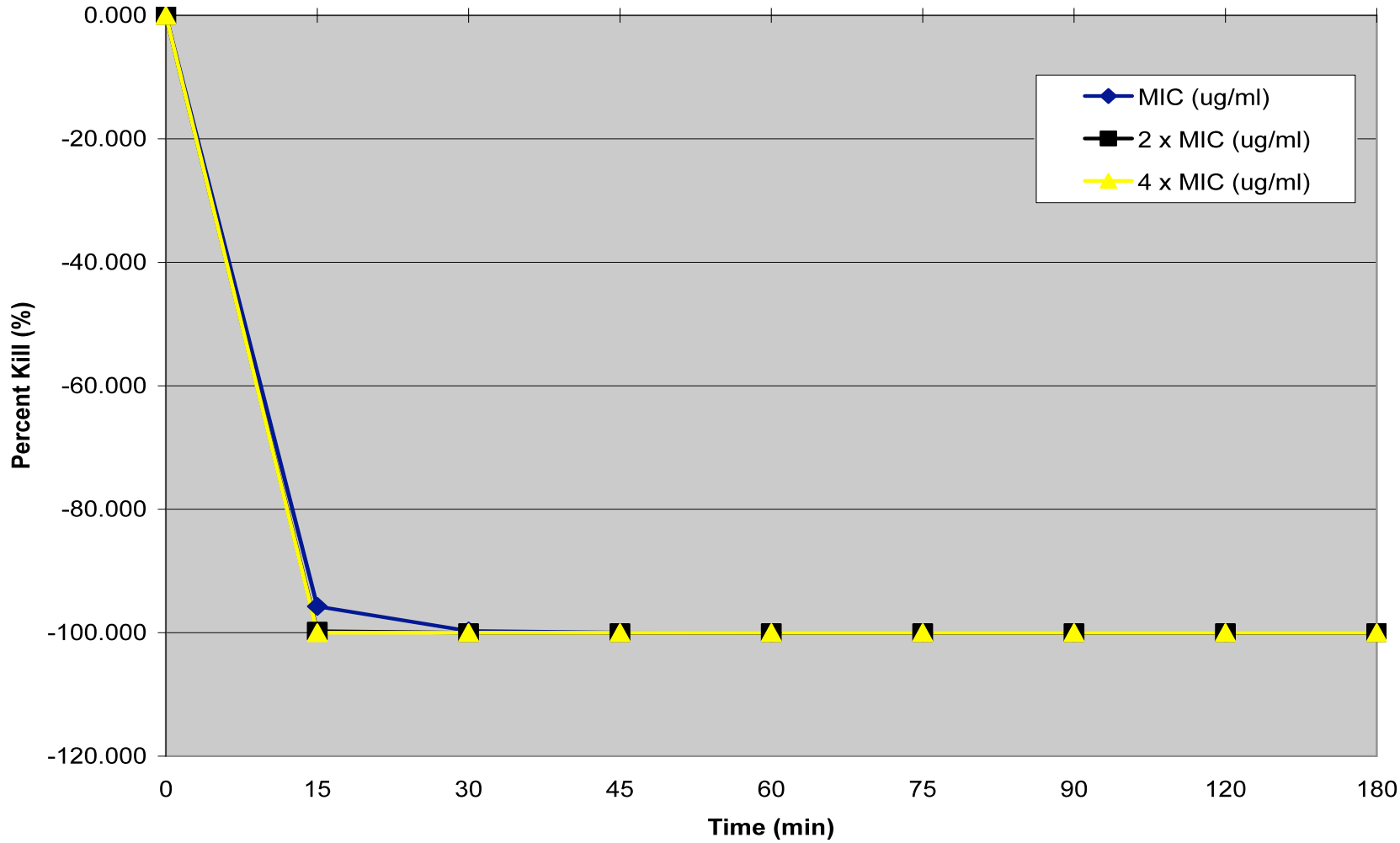


**Figure 3.4.3.2: The Percent Kill (%) of viable cells using different concentrations of alexidine against clinical isolates of MRSA (n = 3)**
MIC = minimum inhibitory concentration; MRSA = Methicillin-resistant *Staphylococcus aureus*

| Time (min) | $Log_{10}$-Reduction of Viable Cells | | |
|---|---|---|---|
| | Alexidine Concentration | | |
| | MIC (µg/ml) | 2 x MIC (µg/ml) | 4 x MIC (µg/ml) |
| **0** | 0.000 | 0.000 | 0.000 |
| **15** | -1.371 | -2.607 | -4.217 |
| **30** | -2.565 | -3.929 | -5.217 |
| **45** | -3.857 | -5.230 | -5.217 |
| **60** | -4.556 | -5.230 | -5.217 |
| **75** | -4.778 | -5.230 | -5.217 |
| **90** | -5.079 | -5.230 | -5.217 |
| **120** | -5.079 | -5.230 | -5.217 |
| **180** | -5.079 | -5.230 | -5.217 |

**Table 3.4.3.3: The $Log_{10}$-reduction of viable cells using different concentrations of alexidine against one clinical isolate of MSSA**
MIC = minimum inhibitory concentration; MSSA = Methicillin-susceptible *Staphylococcus aureus*



| Time (min) | Percent Kill (%) | | |
|---|---|---|---|
| | Alexidine Concentration | | |
| | MIC (µg/ml) | 2 x MIC (µg/ml) | 4 x MIC (µg/ml) |
| **0** | 0.000 | 0.000 | 0.000 |
| **15** | -95.750 | -99.753 | -99.994 |
| **30** | -99.728 | -99.988 | -100.000 |
| **45** | -99.986 | -100.000 | -100.000 |
| **60** | -99.997 | -100.000 | -100.000 |
| **75** | -99.998 | -100.000 | -100.000 |
| **90** | -99.999 | -100.000 | -100.000 |
| **120** | -99.999 | -100.000 | -100.000 |
| **180** | -99.999 | -100.000 | -100.000 |

**Table 3.4.3.4: The Percent Kill (%) of viable cells using different concentrations of alexidine against one clinical isolate of MSSA**
MIC = minimum inhibitory concentration; MSSA = Methicillin-susceptible *Staphylococcus aureus*

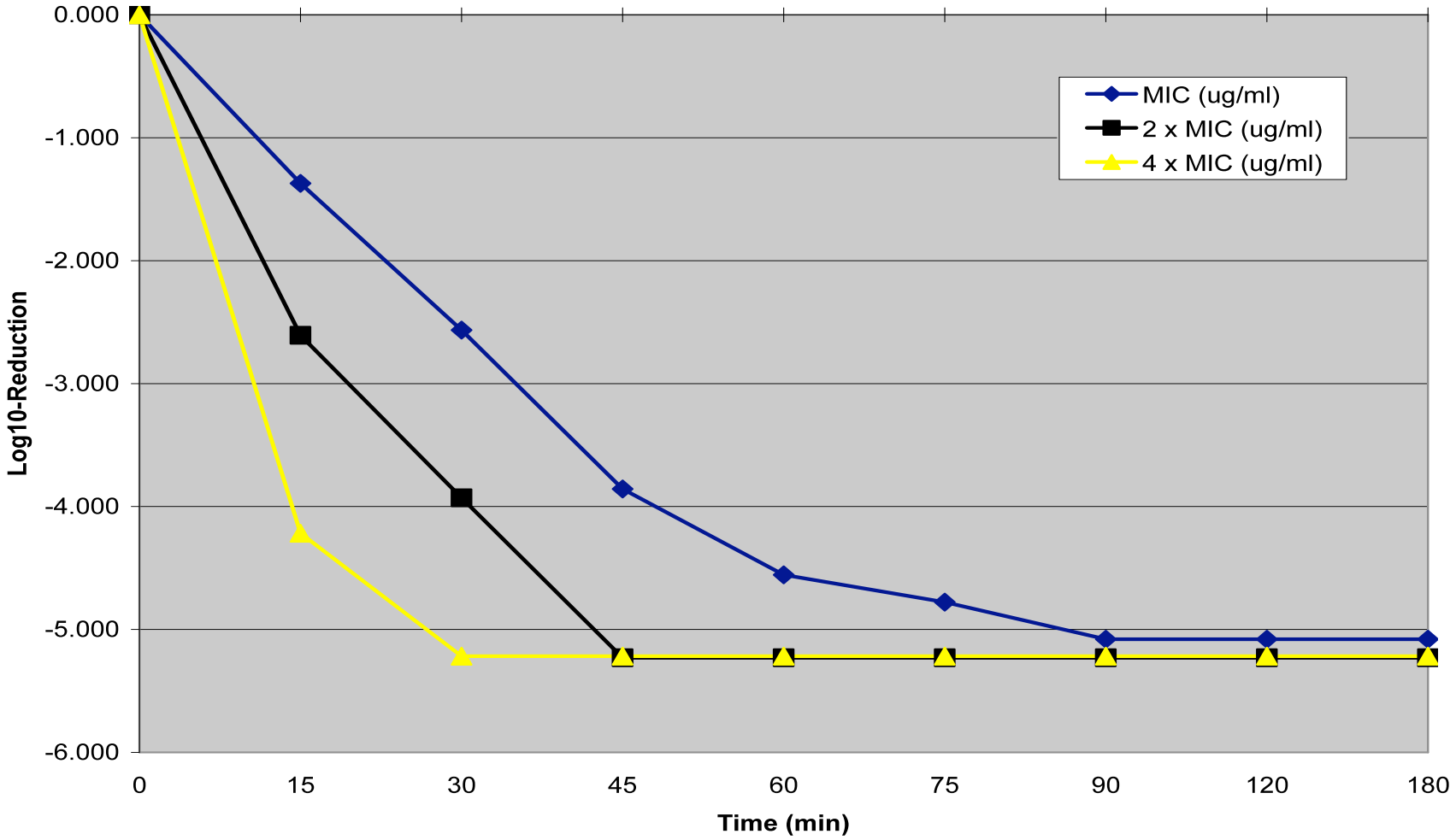


**Figure 3.4.3.3: The $Log_{10}$-reduction of viable cells using different concentrations of alexidine against one clinical isolate of MSSA**
MIC = minimum inhibitory concentration; MSSA = Methicillin-susceptible *Staphylococcus aureus*

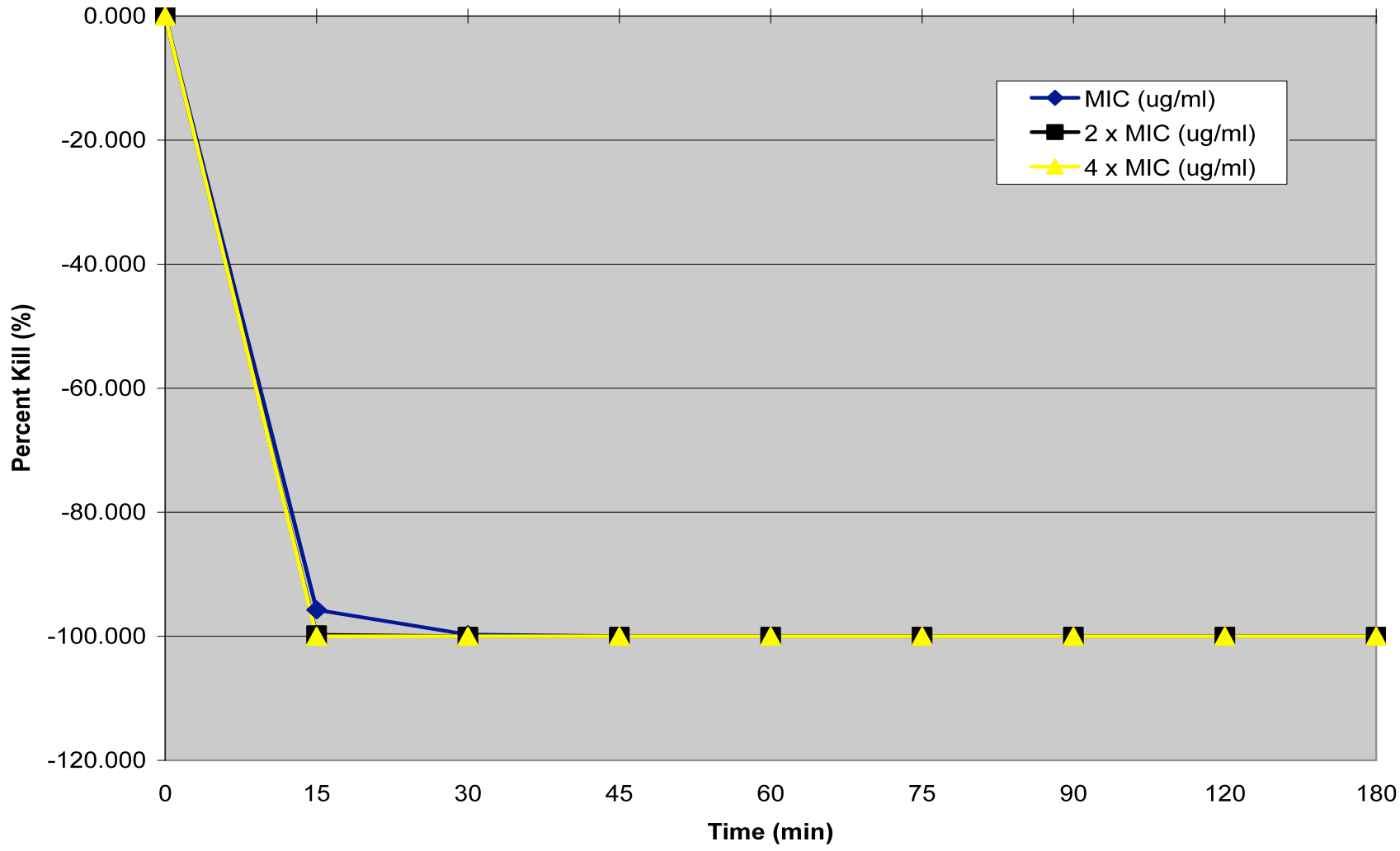


**Figure 3.4.3.4: The Percent Kill (%) of viable cells using different concentrations of alexidine against one clinical isolate of MSSA**
MIC = minimum inhibitory concentration; MSSA = Methicillin-susceptible *Staphylococcus aureus*

| Time (min) | $Log_{10}$-Reduction of Viable Cells | | |
|---|---|---|---|
| | Alexidine Concentration | | |
| | MIC (µg/ml) | 2 x MIC (µg/ml) | 4 x MIC (µg/ml) |
| **0** | 0.000 | 0.000 | 0.000 |
| **15** | -2.301 | -4.048 | -5.834 |
| **30** | -4.307 | -6.334 | -6.491 |
| **45** | -5.516 | -6.334 | -6.491 |
| **60** | -6.057 | -6.334 | -6.491 |
| **75** | -6.057 | -6.334 | -6.491 |
| **90** | -6.057 | -6.334 | -6.491 |
| **120** | -6.057 | -6.334 | -6.491 |
| **180** | -6.057 | -6.334 | -6.491 |

**Table 3.4.3.5: The $Log_{10}$-reduction of viable cells using different concentrations of alexidine against clinical isolates of *S. pneumoniae* (n = 3)**

MIC = minimum inhibitory concentration



| Time (min) | Percent Kill (%) | | |
|---|---|---|---|
| | Alexidine Concentration | | |
| | MIC (µg/ml) | 2 x MIC (µg/ml) | 4 x MIC (µg/ml) |
| **0** | 0.000 | 0.000 | 0.000 |
| **15** | -99.302 | -99.988 | -100.000 |
| **30** | -99.990 | -100.000 | -100.000 |
| **45** | -99.999 | -100.000 | -100.000 |
| **60** | -100.000 | -100.000 | -100.000 |
| **75** | -100.000 | -100.000 | -100.000 |
| **90** | -100.000 | -100.000 | -100.000 |
| **120** | -100.000 | -100.000 | -100.000 |
| **180** | -100.000 | -100.000 | -100.000 |

**Table 3.4.3.6: The Percent Kill (%) of viable cells using different concentrations of alexidine against clinical isolates of *S. pneumoniae* (n = 3)**

MIC = minimum inhibitory concentration

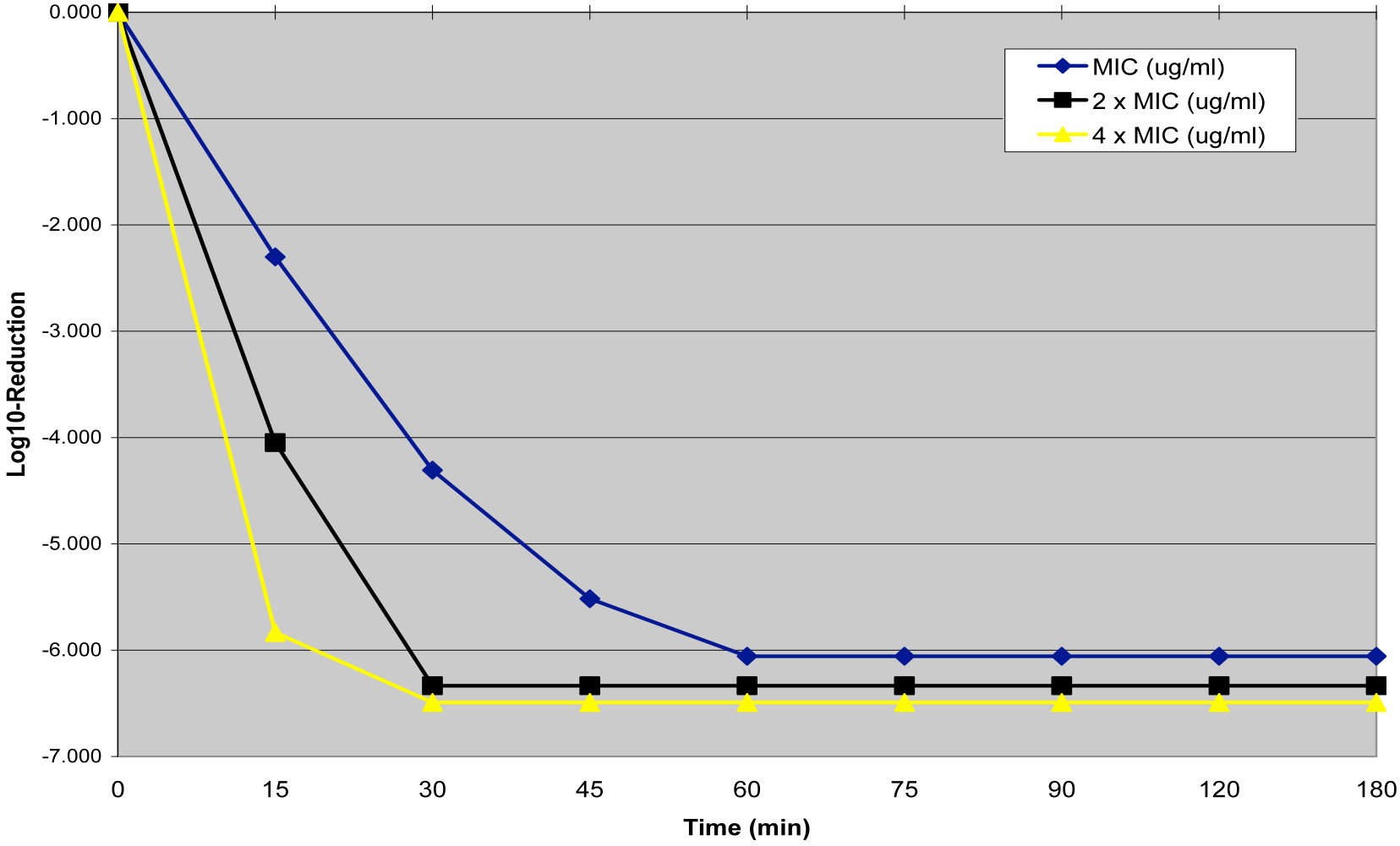


**Table 3.4.3.5: The $Log_{10}$-reduction of viable cells using different concentrations of alexidine against clinical isolates of *S. pneumoniae* (n = 3)**

MIC = minimum inhibitory concentration

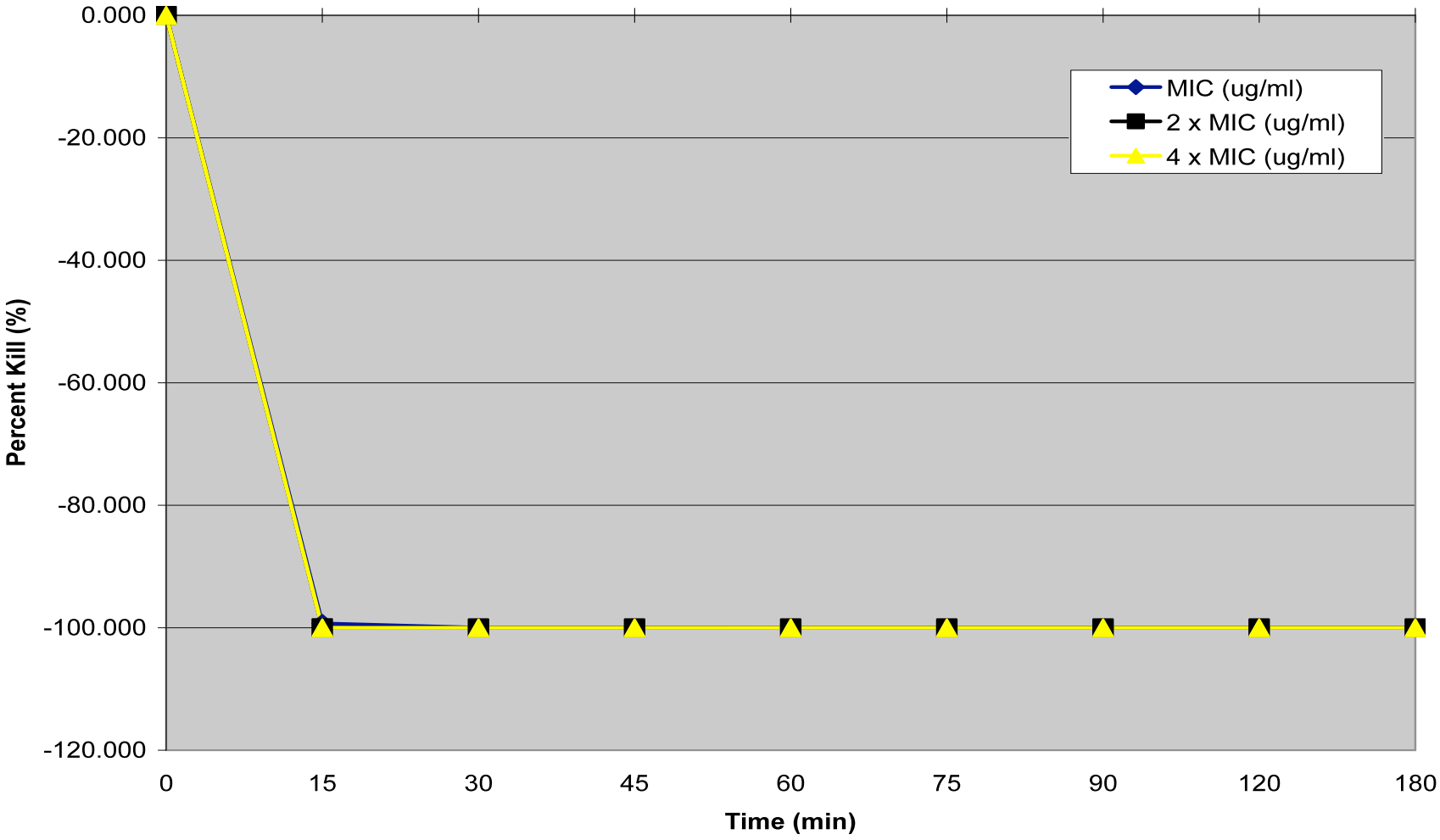


**Figure 3.4.3.6: The Percent Kill (%) of viable cells using different concentrations of alexidine against clinical isolates of *S. pneumoniae* (n = 3)**

MIC = minimum inhibitory concentration

| Time (min) | $Log_{10}$-Reduction of Viable Cells | | |
|---|---|---|---|
| | Alexidine Concentration | | |
| | MIC (µg/ml) | 2 x MIC (µg/ml) | 4 x MIC (µg/ml) |
| **0** | 0.000 | 0.000 | 0.000 |
| **15** | -4.25 | -6.487 | -6.538 |
| **30** | -5.773 | -6.743 | -6.538 |
| **45** | -6.84 | -6.743 | -6.538 |
| **60** | -6.84 | -6.743 | -6.538 |
| **75** | -6.84 | -6.743 | -6.538 |
| **90** | -6.84 | -6.743 | -6.538 |
| **120** | -6.84 | -6.743 | -6.538 |
| **180** | -6.84 | -6.743 | -6.538 |

**Table 3.4.3.7: The $Log_{10}$-reduction of viable cells using different concentrations of alexidine against clinical isolates of *P. aeruginosa* (n = 2)**

MIC = minimum inhibitory concentration



| Time (min) | Percent Kill (%) | | |
|---|---|---|---|
| | Alexidine Concentration | | |
| | MIC (µg/ml) | 2 x MIC (µg/ml) | 4 x MIC (µg/ml) |
| **0** | 0.000 | 0.000 | 0.000 |
| **15** | -99.994 | -100.000 | -100.000 |
| **30** | -100.000 | -100.000 | -100.000 |
| **45** | -100.000 | -100.000 | -100.000 |
| **60** | -100.000 | -100.000 | -100.000 |
| **75** | -100.000 | -100.000 | -100.000 |
| **90** | -100.000 | -100.000 | -100.000 |
| **120** | -100.000 | -100.000 | -100.000 |
| **180** | -100.000 | -100.000 | -100.000 |

**Table 3.4.3.8: The Percent Kill (%) of viable cells using different concentrations of alexidine against clinical isolates of *P. aeruginosa* (n = 2)**

MIC = minimum inhibitory concentration

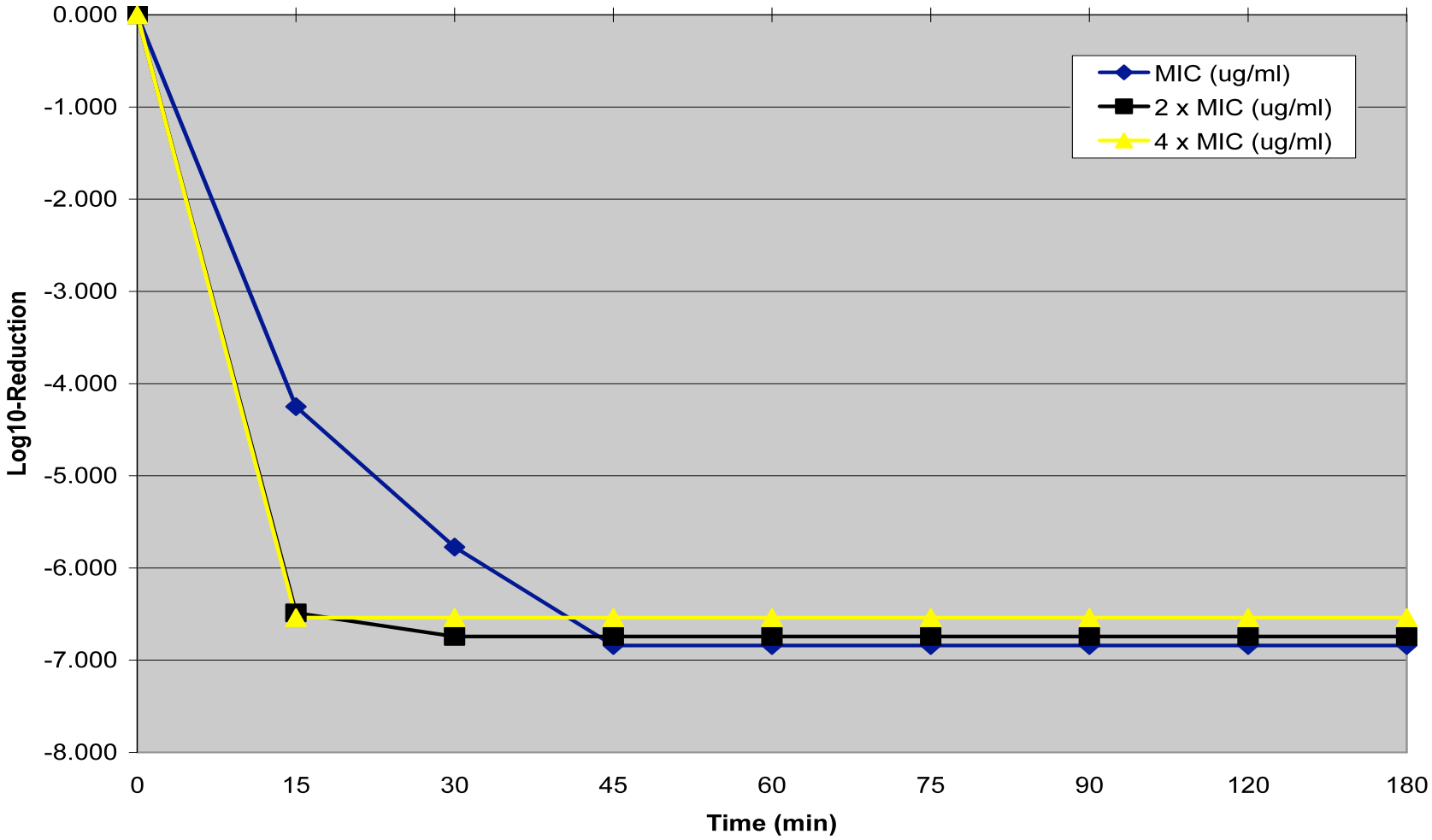


**Figure 3.4.3.7: The $Log_{10}$-reduction of viable cells using different concentrations of alexidine against clinical isolates of *P. aeruginosa* (n = 2)**

MIC = minimum inhibitory concentration

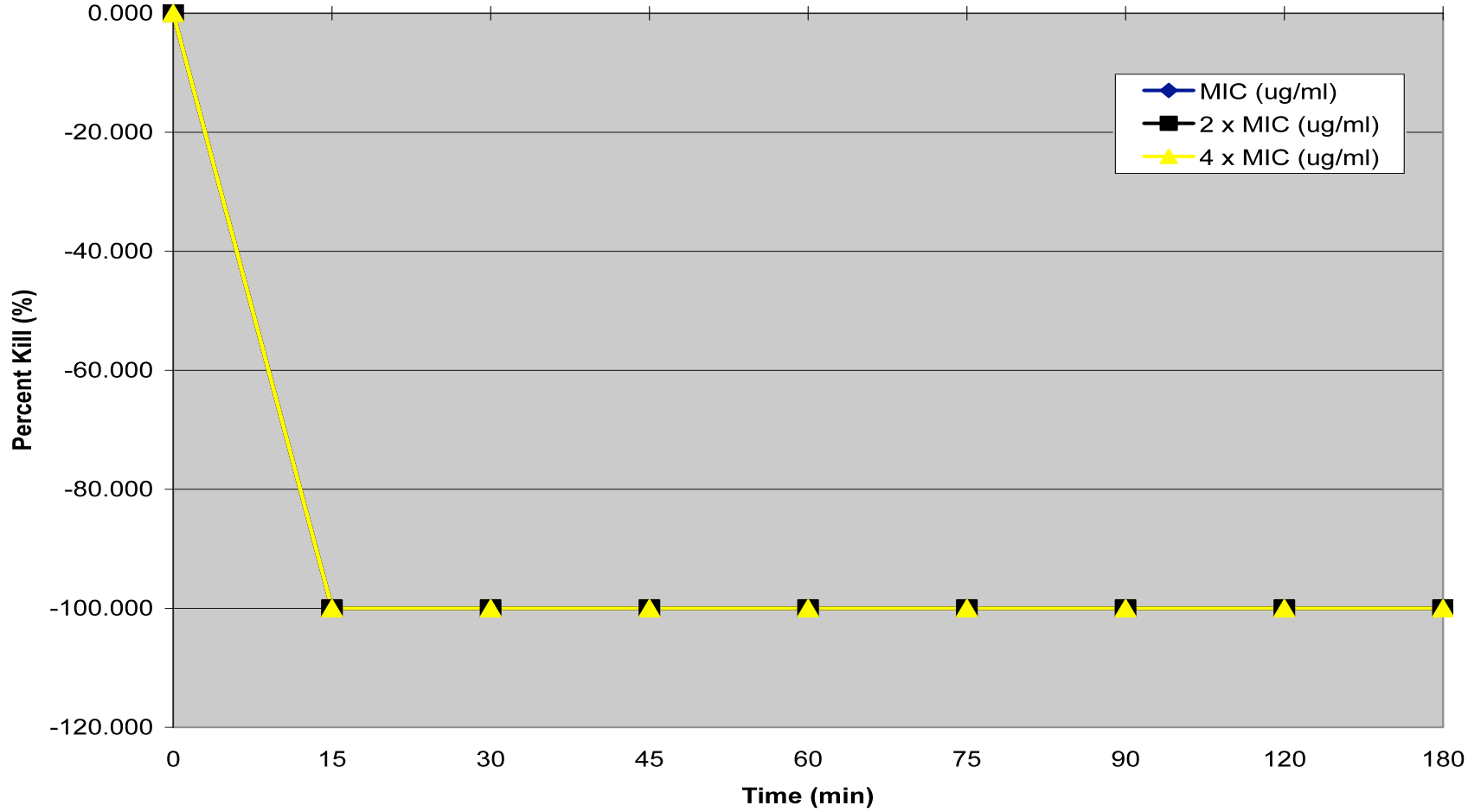


**Figure 3.4.3.8: The Percent Kill (%) of viable cells using different concentrations of alexidine against clinical isolates of *P. aeruginosa* (n = 2)**

MIC = minimum inhibitory concentration

## 4.0 DISCUSSION

### 4.1 Methods to Simplify MPC Testing

The MPC method is potentially an important tool for the clinical microbiology laboratory because it can give a measured indication of the possibility or extent of resistance selection. Unfortunately, the present protocol for MPC testing is too tedious and time-consuming to be used routinely in clinical microbiology susceptibility testing. In an attempt to make MPC testing more feasible for the clinical laboratory, we developed two new MPC methods: the microbroth dilution method and the E-test method. We also looked at the possibility of using linear regression as a method for extrapolating MPC values from experimentally measured MIC values.

The modified microbroth dilution method, developed in our laboratory, is less time-consuming and labour-intensive than the traditional agar dilution method [78]. The microbroth dilution method requires a small volume (10 ml) of high-density bacterial inoculum for all organisms while the traditional MPC testing of *S. aureus* requires 100 ml of high-density bacterial suspension and traditional MPC testing of *S. pneumoniae* requires 500 ml of high-density bacterial suspension. An incubation period of two hours, under appropriate conditions, is needed to achieve a bacterial density of $\geq 10^9$ CFU/ml in a 10 ml suspension, whereas 18 to 24 hours of incubation under appropriate conditions are required when growing a bacterial suspension of ≥100 ml up to a bacterial density of $\geq 10^9$ CFU/ml.

An advantage of the novel microbroth dilution MPC method is that inhibitory values (μg/ml) can be determined for different bacterial concentrations simultaneously. The result of performing consecutive 1:10 (700 μl into 7 ml) dilutions on the high-density

bacterial inoculum, following the two hour incubation under appropriate conditions, is a series of bacterial suspensions each with a different concentration (i.e. $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$, and $10^9$ CFU/ml). By adding each of these dilutions to a separate row in a prepared 96-well microtitre panel (containing doubling dilutions of the antimicrobial agent of interest), it is possible to determine the concentration of antimicrobial agent required to inhibit different bacterial burdens. For each row, the lowest drug concentration with no visible growth is recorded as the inhibitory concentration. The bacterial burden of $10^5$ CFUs corresponds to the MIC and the bacterial burden of $10^9$ CFUs ($10^7$ CFUs for *S. pneumoniae*) corresponds to the MPC. The microbroth dilution method is therefore able to simultaneously determine MIC and MPC values for specific pathogen/antimicrobial agent combinations.

The microbroth dilution method shortens the time needed for MPC testing from five day to three days, making the microbroth dilution method more comparable to MIC testing in terms of assay duration. In our experiments, we measured consistent MPC values using either the traditional- or the modified microbroth dilution- method [78]. This finding suggests that the modified microbroth dilution method is a reliable method for determining MPC values; however, extension of this method to more bacteria-antimicrobial agent combinations is necessary to further validate this procedure.

The E-test method is commonly used in clinical laboratories as a fast and simple way of determining MIC values; we looked at the E-test as a possible method for determining MPC values. The only real difference in determining an MIC value versus determining an MPC value is the bacterial density being used in the assay. In MIC testing, bacterial density of $10^5$ CFU/ml is used, whereas in MPC testing a bacterial

density of $\geq 10^9$ CFU/ml is used. Therefore, MPC testing using the E-test method is very similar to MIC testing using the E-test method; except a higher density bacterial suspension is placed on the agar plate prior to the addition of the E-test strip in MPC testing.

Unfortunately, MPC values measured using the E-test method were not consistent with MPC values measured using the traditional agar dilution method. The MPC values measured using the E-test method were substantially lower than the MPC values measured using the traditional agar dilution method. The probable explanation for this lies in the fact that spontaneous mutations occur at a rate of $1x10^{-7}$ to $1x10^{-9}$ [68]. Therefore, in a bacterial burdenof $10^9$ CFUs, there may only be one or two mutants present. The probabiliy of those mutants being positioned right along side the E-test strip on the agar plate is very low. For this reason, the E-test is not an appropriate method for determining MPC values.

We used linear regression models to determine whether it would be possible to extrapolate MPC values from experimentally determined MIC values for particular bacterial pathogens against particular antimicrobial agents. Correlation coefficients ($r^2$ values) were calculated for several fluoroquinolones against five bacterial species and for three macrolides against *S. pneumoniae* using data from published and unpublished studies of clinical isolates. We observed that MPC values and MIC values correlated poorly with the majority of $r^2$ values falling below 0.5 [82].

Low correlations between MIC and MPC values are likely to require a complex explanation. Some of these isolates probably contain mutant subpopulations that vary considerably in relative abundance and drug susceptibility, which will contribute to a

wide variation in MIC values [82]. In fact, we have found isolates with the same MIC value whose MPC values have ranged over five twofold dilutions. Unfortunately, because MPC values cannot be estimated accurately from MIC values, measurement of MPC values will continue to be required for each individual clinical isolate.

While the E-test method and linear regression models do not appear to be appropriate methods for the determination of MPC values, the microbroth dilution method does appear to be a valuable method for MPC testing. The microbroth dilution method represents an important advancement in MPC testing because it is less technically demanding and time-consuming than the currently available method; the agar dilution method. Hopefully this improved novel method (microbroth dilution method) will ultimately allow for the facilitation of MPC testing in susceptibility testing in clinical microbiology laboratories.

**4.2 The Impact of Media on MPC Testing**

While conducting MPC testing with clinical isolates of *S. pneumoniae* against tigecycline we noticed unusually high MPC values. The $MPC_{90}$ values we measured for *S. pnuemoniae* isolates against tigecyline were ≥500-fold higher than the corresponding measured $MIC_{90}$ values. The MPC values for most bacteria-antimicrobial agent combinations are generally 2 to 8 fold greater than the corresponding MIC values [83]. After further testing we observed that the abnormally high MPC values were only measured when using media containing blood [80, 81]. MPC testing of tigecycline against MRSA and MSSA did not yield elevated MPC values in the presence of blood indicating the phenomenon may be limited to MPC testing of tigecycline against *S. pneumoniae* [84].

It is widely known that different bacterial organisms require different conditions for optimal growth. Some of the factors that have the potential to affect the growth of bacterial organisms include: incubation conditions (temperature, atmosphere, light, duration of incubation, etc.), composition and pH of media (i.e. presence of blood), and the protocol used [64]. The Clinical and Laboratory Standards Institute (CLSI) has set forth guidelines to ensure that optimal growth conditions for bacterial isolates and proper preparation of antimicrobial agents is standardized in every clinical laboratory. For example, CLSI guidelines state susceptibility testing of *S. pneumoniae* should be done on media containing blood while susceptibility testing of *Staphylococci* spp. does not require blood; *S. pneumoniae* should also be incubated in $CO_2$ while *Staphylococci* spp. should be incubated in ambient air ($O_2$) [85, 86]. Unfortunately, the CLSI does not provide guidelines for standardized MPC testing.

The results of our experiments with *S. pneumoniae* and tigecycline indicate that MPC testing of *S. pneumoniae* against tigecycline should be avoided on media containing blood as it yields falsely elevated MPC values. At this point, we do not have an explanation for the elevated MPC values observed in MPC testing of tigecycline against *S. pneumoniae* on blood. Molecular experiments are likely to be required in order to elucidate the actual interaction between *S. pneumoniae*, tigecycline, and blood. We speculate that the haemolytic quality of *S. pneumoniae* may be a factor in the observed interaction. Our observations illustrate the profound effect media choice may have on susceptibility results. It would be extremely beneficial to have standardized guidelines for MPC testing of various bacterial pathogens and antimicrobial agents as exists for MIC testing. It is our hope that eventually bodies such as the CLSI in North America and the

European Committee on Antimicrobial Susceptibility Testing (EUCAST) in Europe will set forth MPC testing guidelines and perhaps ultimately MPC breakpoints.

**4.3 The Use of BAK in Conjunction with gatifloxacin and moxifloxacin**

We conducted studies using BAK in conjunction with two commonly used ophthalmic fluroquinolones; gatifloxacin and moxifloxacin. Both agents, Vigamox® (Alcon, Inc.) and Zymar® (Allergan, Inc.), are currently being used in competing commercial preparations for the treatment of bacterial conjunctivitis. Vigamox® is a formula containing 0.5% moxifloxacin while Zymar® is a formula containing 0.3% gatifloxacin plus 0.005% BAK. One of the main differences between Vigamox® and Zymar® is the addition of BAK to Zymar®. There has been much debate over which product, Vigamox® or Zymar®, is more effective in the treatment of bacterial conjunctivitis [48, 87].

Several studies have been conducted, both *in vitro* and *in vivo*, to determine which fourth generation fluoroquinolone, gatifloxacin or moxifloxacin, is better in terms of its ocular toxicity and antimicrobial activity. The findings of these studies vary widely. In recent *in vitro* studies, Sosa *et al* used corneal and conjunctival epithelial cells to look at the toxicity of gatifloxacin and moxifloxacin; they found that gatifloxacin was more toxic than moxifloxacin in both cell types [88]. Baez *et al* made similar observations using a mouse model; gatifloxacin was more toxic than moxifloxacin [89]. However, using a rabbit model, Herrygers *et al* found no difference in epithelial damage between gatifloxacin and moxifloxacin [90]. In human studies, Donnenfeld *et al*, reported that patients who received gatifloxacin reported better tolerability than patients who received moxifloxacin [91].

We conducted *in vitro* studies to look at the antimicrobial activity of gatifloxacin and moxifloxacin. We measured similar $MIC_{90}$ values (within one doubling dilution) for either antimicrobial agent (gatifloxacin or moxifloxacin) against *Staphylococci* spp., *P. aeruginosa*, and *E. coli*. However, we did measure different $MIC_{90}$ values for gatifloxacin and moxifloxacin against *S. pneumoniae*; 1 µg/ml and 0.125 µg/ml respectively (Section 3.3.1). Our studies show that the antimicrobial activity of moxifloxacin is better (lower MIC value) against *S. pneumoniae* than gatifloxacin, but both agents have similar antimicrobial activity against the other pathogens we tested. Interestingly, in a recent retrospective cross-sectional study, Jensen *et al* found that the incidence of postoperative bacterial endophthalmitis was significantly higher in patients who had received moxifloxacin than in those who had received gatifloxacin in cataract surgery [87]. It is evident that further studies need to be conducted to determine which antimicrobial agent should be the agent of choice. The debate as to which antimicrobial agent is "better" remains ongoing.

To determine if the addition of BAK to the commercial preparation of Zymar® contributes to the clinical effect, we performed MIC and MPC experiments as well as time-kill assays with BAK alone and in combination with gatifloxacin and/or moxifloxacin. Results from MIC studies indicate that the combination of gatifloxacin or moxifloxacin with BAK is more active (lower MIC values) than either fluoroquinolone without BAK. In fact, $MIC_{90}$ values for gatifloxacin plus BAK and moxifloxacin plus BAK against clinical isolates of MRSA were greater than 1000-fold lower than $MIC_{90}$ values for gatifloxacin or moxifloxacin alone. $MIC_{90}$ values for gatifloxacin plus BAK and moxifloxacin plus BAK against all other clinical isolates tested (MSSA, CNS, *S.*

*pnuemoniae*, *P. aeruginosa*, and *E. coli*) were 4 to 500-fold lower than $MIC_{90}$ values for gatifloxacin or moxifloxacin alone. Furthermore, the actual concentration of BAK required to achieve such substantial reductions in MIC values is less than the concentration of BAK present in Zymar®, except for *P. aeruginosa*. The concentration of BAK present in Zymar® is 50 μg/ml (0.005%), however, the concentration of BAK required to obtain the 1000-fold decrease in $MIC_{90}$ values observed when used in conjunction with gatifloxacin and moxifloxacin against MRSA is 3.125 μg/ml [24]. Similar observations were made in MPC tests with BAK in conjunction with gatifloxacin and moxifloxacin; BAK lowered the MPC values of gatifloxacin and moxifloxacin against clinical isolates of MRSA. [19]. These findings indicate that BAK has a clinical impact on the MIC and MPC values of both Gram-positive and Gram-negative organisms *in vitro*. The mechanism of this enhanced *in vitro* activity remains unknown; however, one possibility is that BAK increases the facilitation of gatifloxacin or moxifloxacin into the bacterial cell. Recall, BAK exerts its effect by causing the dissociation of the bacterial lipid bilayer [51]. This dissociation may provide "easy access" for gatifloxacin or moxifloxacin to enter the cell and exert its effect; inhibition of bacterial DNA synthesis [19].

Time-kill assays were performed using combinations of gatifloxacin and moxifloxacin with and without the addition of BAK. First, studies were conducted to look at the killing action of BAK alone. As expected, the higher the concentration of BAK, the greater the $\log_{10}$-reduction and percent kill of viable cells. At a concentration of 50 μg/ml, the concentration of BAK in Zymar®, there was a greater than 5-fold decrease in viable cells 10 minutes after the addition of BAK to clinical MRSA isolates.

This 5-fold decrease corresponded to a 100% kill of all viable cells. The killing power of BAK decreased with decreasing concentration of BAK, indicating that BAK is a concentration dependent antimicrobial agent.

In time-kill assays looking at the action of BAK alone and in conjunction with gatifloxacin against clinical MRSA isolates, there was a marked decrease in viable cells when BAK was used in conjunction with gatifloxacin compared to gatifloxacin alone. The concentration of BAK was arbitrarily chosen to be 25 μg/ml and the concentration of gatifloxacin corresponded to the measured MIC values. Interestingly, the combination of gatifloxacin plus BAK did not provide much better killing action than BAK alone. The percent kill of viable MRSA cells 180 minutes after the addition of antimicrobial agent(s) was -99.99% for BAK alone and -100% for gatifloxacin plus BAK, compared to -76.08% for gatifloxacin alone. This observation may be a result of the high concentration of BAK used (25 μg/ml). The $MIC_{90}$ of BAK against clinical isolates of MRSA is 3.125 μg/ml. If this study were to be conducted again, an antimicrobial agent concentration corresponding to the MIC should be used for both BAK and gatifloxacin. With such a high concentration of BAK, it is possible that the killing-effect observed is not due to the combination of gatifloxacin plus BAK but due to BAK itself.

Additional time-kill assays were conducted that perhaps more accurately reflect the actual concentrations of BAK and gatifloxacin administered to the eye when using Zymar®. Zymar® is a topical ophthalmic agent that is available as eye drops. The concentrations of gatifloxacin and BAK in Zymar® are 3,000 μg/ml and 50 μg/ml respectively and one drop of Zymar® has an approximate volume of 100 μl. Therefore, the approximate amounts of gatifloxacin and BAK administered to the eye in one drop of

Zymar® are 300 μg and 5 μg respectively. In a time-kill assay using concentrations of 300 μg gatifloxacin and 5 μg BAK against clinical MRSA isolates, the percent kill 15 minutes after the addition of antimicrobial agent(s) was -53.89 % for gatifloxacin alone, -51.65% for gatifloxacin plus BAK, -59.09% for Zymar®, and -9.30% for BAK alone. These results indicate that BAK alone has very little killing activity at a concentration of 5 μg/ml in the first 15 minutes after its addition to a bacterial population of MRSA. Interestingly, at a concentration of 5 μg/ml, the killing-action of gatifloxacin plus BAK against MRSA is similar to the killing-action of gatifloxacin alone. This suggests that longer exposure to low BAK concentrations may be necessary to generate the high kill percentage observed when using higher concentrations of BAK.

The prescribed use of Zymar® is one drop in the infected eye every two hours [92]. However, anecdotal and personal experience suggests that the delivery of only one drop to the eye is difficult and often more than one drop is administered. If two drops of Zymar® were inadvertently administered to the eye, the actual amount of gatifloxacin and BAK delivered to the eye would be 600 μg and 10 μg respectively. In time-kill studies using a concentration of 300 μg/ml gatifloxacin plus 10 μg/ml BAK against clinical isolates of MRSA, Blondeau *et al* measured a percent kill of -55.20% 15 minutes after the addition of antimicrobial agents. Blondeau *et al* also measured the percent kill of 300 μg/ml gatifloxacin plus 15 μg/ml BAK and 300 μg/ml gatifloxacin plus 20 μg/ml BAK against clinical isolates of MRSA; -66.43% and 92.73% respectively 15 minutes after the addition of antimicrobial agents (Blondeau, *et al.*, unpublished data, personal communication). These studies hint toward the effect we would observe if 600 μg/ml of

gatifloxacin plus 10 μg/ml BAK were delivered to the eye (as they would be if two drops were administered at once); the killing activity of Zymar® would be enhanced.

There is no doubt that BAK is a potent killer of the bacterial pathogen MRSA at high concentrations, however, it does not appear to contribute to the actual killing-activity of Zymar® at concentrations that would be administered to the eye if one drop is used. We have shown, however, that lower concentrations of BAK are able to substantially lower the MIC values of gatifloxacin and moxifloxacin against Gram-positive and Gram-negative organisms. Recall that 3.125 μg/ml of BAK lowered the MIC of gatifloxacin and moxifloxacin by 1000-fold against clinical isolates of MRSA. This is possible because MIC and MPC are measurements of inhibition of growth and not kill. It is evident that further studies are needed to elucidate the true clinical impact of BAK.

**4.4 Susceptibility Testing of Alexidine**

There is an urgent need for new antimicrobial agents, especially those with activity against both Gram-positive and Gram-negative organisms [14, 43]. Alexidine is an antimicrobial agent that was previously used in the dental industry as an effective disinfectant and is currently being looked at as an agent for use in the field of ophthalmology [44]. Fluoroquinolones are currently the antimicrobial agent of choice in the ophthalmic community, however, as resistance to the fluoroquinolones continues to rise there is a need for new agents with better antimicrobial activity [22, 26, 29-31]. Susceptibility testing of alexidine revealed that it is more active (lower MIC values) against Gram-positive pathogens than against Gram-negative pathogens. Importantly, we also found that alexidine is more active against Gram-positive pathogens, especially

MRSA, than the fluoroquinolones gatifloxacin, moxifloxacin, and levofloxacin. This suggests that alexidine may be a better choice for treating ocular infections than the currently indicated fluoroquinolones.

We were unable to complete traditional MPC testing using alexidine against MRSA or MSSA. When the microbroth dilution method for MPC testing was used we measured $MPC_{90}$ values that were greater than 32-fold higher than the corresponding $MIC_{90}$ values. As previously stated, MPC values are generally 2 to 8 fold higher than MIC values [83]. If the MPC values measured using the microbroth dilution method are accurate then initial susceptibility testing indicates that alexidine may have a high likelihood for selecting resistance based on MPC measurements. However, it is more likely that the MPC results measured for alexidine against MRSA and MSSA are inaccurate as there have been few if any reports of alexidine resistance at use concentrations, in spite of its widespread use for almost 50 years in clinical and domestic settings [16]. It is very unlikely that with such little resistance being reported over the last 50 years that the MPC values are accurate. In support of this conclusion is the abnormally high MPC values measured when alexidine was tested against clinical isolates of *S. pneumoniae*. The $MPC_{90}$ values were greater than 32-fold higher than the corresponding $MIC_{90}$ values for susceptibility testing of alexidine against clinical isolates of *S. pnuemoniae*. Difficulties with MPC testing suggest that it may be necessary to modify the MPC protocol in order to measure MPC values of alexidine against MRSA, MSSA, or *S. pneumoniae*. Such modifications may include different media, broth, and growth conditions.

When time-kill studies were performed, alexidine proved to have very potent killing activity against both Gram-positive and Gram-negative organisms. Substantial bactericidal activity (>3fold $\log_{10}$-reduction) was observed very shortly after the addition of alexidine to bacterial isolates. In fact, we calculated >99.999% kill of viable cells 15 minutes after the addition of alexidine at concentrations corresponding to 2 x MIC and 4 x MIC against clinical isolates of MRSA, MSSA, *S. pneumoniae*, and *P. aeruginosa*.

Initial susceptibility testing of alexidine indicates that it is potentially a very potent antimicrobial agent with good activity against both Gram-positive and Gram-negative organisms.

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# 6.0 APPENDIX A

## 6.1 Solutions and Media

MHB
Add 21 g to 1 L of distilled water and then autoclave

Skim Milk
Add 200 g to 1 L of distilled water and then autoclave

sTHB
Add 15 g of granulated agar and 30 g of THB to 1 L of distilled water and autoclave

THB
Add 30 g to 1 L of distilled water and then autoclave

TSA
Add 40 g to 1 L of distilled water and then autoclave

# 7.0 APPENDIX B

## 7.1 Suppliers

### 7.1.1 Media

| | |
|---|---|
| Chocolate Agar Plates | PML Microbiologicals, Winnipeg, MB |
| Granulated Agar | Becton Dickinson, Sparks, MD |
| Mueller Hinton Broth (MHB) | Becton Dickinson, Sparks, MD |
| Todd Hewitt Broth (THB) | Becton Dickinson, Sparks, MD |
| Tryptic Soy Agar (TSA) | Becton Dickinson, Sparks, MD |
| 5% Sheep Red Blood Cells | Oxoid, Ryegate, MT |

### 7.1.2 Antimicrobial Agents

Alexidine (solution) – Bausch and Lomb Pharmaceuticals Inc., Rochester, NY, U.S.A.

Benzalkonium chloride (BAK) (solution) – Allergan Inc., Irvine, CA, U.S.A.

Gatifloxacin (powder) – Bristol Myers Squibb, Montreal, QC, Canada

Gatifloxacin (E-test)- AbBiodisk, U.S.A.

Gemifloxacin (powder) – Glaxo Smith Kline Pharmaceuticals, Collegeville, PA, U.S.A.; Oscient Pharmaceuticals, Waltham, MA, U.S.A.

Levofloxacin (solution) – Janssen Ortho, Toronto, ON, Canada

Moxifloxacin (powder) – Bayer Pharmaceutical, Toronto, ON, Canada

Tigecycline (powder) – Wyeth Pharmaceuticals, Collegeville, PA, U.S.A

Vigamox® (commercial solution) – Alcon, Fort Worth, TX, U.S.A.

Zymar® (commercial solution) – Allergan Inc., Irvine, CA, U.S.A

### 7.1.3 Reagents and Chemicals

| | |
|---|---|
| 95% Alcohol | Commercial Alcohols Inc., Brampton, ON |
| Saline | Baxter, Deerfield, IL |
| Skim Milk | Becton Dickinson, Sparks, MD |

### 7.1.4 Disposable Labware

| | |
|---|---|
| 10 µl Pipette Tips | Fisher Scientific, U.S.A. |
| 200 µl Pipette Tips | VWR International, Edmonton, AB |
| 5000 µl Pipette Tips | Fisher Scientific, U.S.A. |
| 96-Well Microtitre Panels | Sarstedt, Newton, NC |
| Corning Cryovials | Corning Inc., Corning, NY |
| Cuvettes | Fisher Scientific, U.S.A. |
| Disposable Centrifuge Tube (15 ml) | Fisher Scientific, U.S.A. |
| Glass Tubes | Fisher Scientific, U.S.A. |
| Latex Gloves | Best, Coaticook, QC |
| McFarland Tubes | Fisher Scientific, U.S.A. |
| Pasteur Pipettes | Samco Scientific, U.S.A. |
| Sterile Plastic Petri Plates | Fisher Scientific, U.S.A. |
| Swabs | Puritan, Guilford, ME |
| Wooden Applicator Sticks | Fisher Scientific, U.S.A. |

### 7.1.5 Equipment

| | |
|---|---|
| 10 µl and 300 µl Multichannel Pipettor | Fisher Scientific, U.S.A. |
| 20 µl, 200 µl, and 1 ml Pipettors | Gilson Co., Inc., Lewis Center, OH |
| 5000 µl Pipettor | Fisher Scientific, U.S.A. |
| -70°C Freezer | Forma Scientific Inc., Marjetta, OH |
| Avanti J-E Centrifuge | Beckman Coulter, Palo Alto, CA |
| $CO_2$ Incubator | Forma Scientific Inc., Marjetta, OH |
| Colorimeter | Hach Company, Loveland, CO |
| Oxygen Incubator | Thermo Fisher Scientific, U.S.A. |
| Spectrophotometer | Pharmacia, Cambridge, England |
| Vortex Mixer | Fisher Scientific, U.S.A. |
| Water Bath | Mandel Scientific Co., Guelph, ON |
| Weigh Scale – Mettler PC440 | DeltaRange, Zurich, Switzerland |

# 8.0 APPENDIX C

## 8.1 Preparation of Antimicrobial Agents

Alexidine
This agent is available in solution at 400 μg/ml. Dilute to the desired concentration

Benzalkonium chloride (BAK)
This agent is available in solution at 100,000 μg/ml. Dilute to the desired concentration

Gatifloxacin
Dissolve 0.035 g into 10 ml of sterile distilled water. At 93.3% purity, the final concentration is 3265 μg/ml

Gemifloxacin
Dissolve 0.1 g into 8 ml of sterile distilled water. At 75.4% purity, the final concentration is 9425 μg/ml

Levofloxacin
This agent is available in solution at 25,000 μg/ml. Dilute to the desired concentration

Moxifloxacin
Add 0.02 g to 10 ml of sterile distilled water. At 87.8% purity, the final concentration is 1756 μg/ml

Tigecycline
Add 0.1 g into 10 ml of sterile distilled water. At 100% purity, the final concentration is 10,000 μg/ml

Vigamox®
This agent is a commercial solution available at 0.5%. The active antimicrobial agent is moxifloxacin (5000 μg/ml)

Zymar®

This agent is a commercial solution available at 0.3%. The active antimicrobial agent is gatifloxacin (3000 μg/ml). BAK is present as a preservative 0.005% (50 μg/ml)